Marta Sofia Sampaio de Almada

The endocannabinoid anandamide and endometrial remodelling: importance of cyclooxygenase-2 metabolites

O endocanabinóide anandamida e a remodelação do endométrio: importância dos metabolitos derivados da ciclooxigenase-2

Tese do 3º Ciclo de Estudos Conducente ao Grau de Doutoramento em Ciências Farmacêuticas na especialidade de Bioquímica

Trabalho realizado sob a orientação de

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Under the terms of the "Decreto-lei nº 216/92, de 13 de Outubro", it is declared that the following original articles were prepared in the scope of this dissertation. The author states to have afforded a major contribution to the conceptual design, technical execution of the work, interpretation of the results and manuscript preparation of the works included in this thesis.

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Abstract

Each month, endometrial stromal cells proliferate and differentiate into specialized decidual cells, in a process named decidualization, to allow the establishment of an eventual pregnancy. Uterine regeneration is cyclically imposed by menstruation, miscarriage and parturition. Impaired decidualization may result in pregnancy loss, miscarriage and infertility.

Over the last years, the endocannabinoids have emerged as important mediators in reproduction. In addition, they control biological events such as cell proliferation, differentiation and death, and a deregulation of the eCB signalling has been associated with several pathological processes. The endocannabinoid system (ECS) comprises two G-protein-coupled cannabinoid receptors (CB1 and CB2), their endogenous ligands, the endocannabinoids (eCBs), the membrane transporter and the enzymes responsible for the synthesis and degradation of these compounds.

We have previously demonstrated that the levels of anandamide (AEA), the main eCB, must be tightly regulated in rat decidual tissue and that high levels of this eCB induce apoptosis on rat decidual cells, limiting stromal cell differentiation. Cyclooxygenase-2 (COX-2) is a crucial enzyme for implantation and decidualization. In addition, the oxidative metabolism of AEA, mainly controlled by COX-2, is emerging as a new source of bioactive molecules, the prostamides (PMs). Therefore, in this work, we aimed to explore, on primary cultures of rat decidual cells, not only the role of COX-2 oxidative metabolism on AEA-induced apoptosis but also the underlying molecular mechanisms. It was verified that AEA pro-apoptotic effects were mediated by COX-2 oxidative metabolism, and this enzyme was also highlighted as a key metabolic controller of AEA levels during decidual remodelling. Moreover, it was demonstrated that prostamides may be considered novel lipid mediators in the control of cell fate. Hence, we hypothesized that a disruption on eCB signalling, may favor the production of COX-2-mediated metabolites of AEA and overwhelm decidual stability.

Decidualization is orchestrated by a complex network of growth factors, cytokines, interleukins, hormones and other molecules, in which lipids as eCBs and COX-2-derived prostaglandins (PGs) may play a central role. However, the molecular mechanisms that underpin this process in human reproduction are still not identified. In this way, the other aim of this project was to explore the role of AEA on cell proliferation, differentiation and apoptosis, in a human endometrial cell line and in human decidual fibroblats derived from term placenta. The biochemical machinery of the ECS was characterized and it was further disclosed that AEA inhibited endometrial stromal cell proliferation and the

differentiation process. The anti-proliferative effect of AEA was observed in both non-differentiated and differentiated cells and was associated with appearance of polyploidy. This event was independent of cannabinoid receptor 1 (CB1) activation and appeared to be related to a direct interference of AEA on the process of cytoplasmic division.

As endocannabinoids, PGs may be used as biomarkers of uterine receptivity and embryo implantation. Nevertheless, this knowledge has not yet been clinically translated mostly because of the difficulties to quantify PGs *in vivo*. In order to overcome this hurdle, it was developed and validated a sensitive ultra-performance liquid chromatography-mass spectrometry (UPLC-MS/MS) based method to quantify both PGE_2 and $PGF_{2\alpha}$ in cell culture medium. Using the developed methodology, it was demonstrated for the first time, the intimate dialogue between eCBs and PGs, two pivotal lipid molecules during endometrial stromal-derived cells differentiation *in vitro*. In addition, AEA was proposed as a novel mediator of the decidualization process through down-regulation of COX-2 expression and inhibition of PGE_2 release. These effects were dependent on CB1 activation. Interestingly, it was also found an inverse correlation between AEA and PGE_2 levels that may be essential for the onset of stromal cell differentiation. Altogether these findings further highlight endocannabinoids and prostaglandins as potential biomarkers of women (in)fertility.

It has been postulated that a tight regulation of AEA differentially controls cell fate, depending on the development stage of endometrium, environmental context and cell type. In this regard, other aim of this work was to study the impact of a deregulation of AEA signalling in human endometrial stromal cells. It was observed, for the first time, the occurrence of apoptosis in non-differentiated cells associated with the oxidative metabolism of AEA by COX-2. This finding highlights the importance of a balance AEA tone for the endometrial cell proliferation a crucial process for stromal cell differentiation.

In conclusion, this work greatly contributed to the understanding of the molecular basis governing endometrial turnover and differentiation. Alterations on AEA signalling and/or on the cross-talk between AEA and PGE_2 during human decidualization may impair basic cellular process involved in endometrial remodelling, which may ultimately account for aberrant decidual response, pregnancy loss, miscarriage and infertility.

Keywords: endocannabinoid system, anandamide, endometrium, decidualization, apoptosis

Resumo

Todos os meses, as células do estroma uterino proliferam e diferenciam-se em células deciduais, um processo designado de decidualização, que tem como objectivo preparar o útero para uma eventual gravidez. A regeneração do endométrio ocorre após a menstruação, aborto ou parto. Um comprometimento do processo de decidualização pode estar relacionado com aborto espontâneo, infertilidade e distúrbios da gravidez.

Nos últimos anos, os endocanabinóides surgiram como importantes mediadores na reprodução. Estes controlam eventos biológicos, tais como a proliferação celular, diferenciação e apoptose. A desregulação das suas vias de sinalização tem sido associada a vários processos patológicos. O sistema endocanabinóide (ECS) é composto por dois recetores acoplados à proteína-G (CB1 e CB2), os seus ligandos endógenos, os endocanabinóides (eCBs), um transportador membranar e enzimas responsáveis pela síntese e degradação destes compostos.

No nosso laboratório, foi demonstrado que os níveis do principal endocanabinóide, a anandamida (AEA), são altamente regulados no tecido decidual do rato, e que, em culturas primárias de células deciduais de rato, a AEA induz morte celular por apoptose, limitando assim o processo da diferenciação das células do estroma. A ciclo-oxigenase-2 (COX-2) é uma enzima essencial para a implantação e a decidualização. Além disso, o metabolismo oxidativo da AEA, controlada pela COX-2 é considerado uma fonte de moléculas bioactivas, as prostamides (PMs). Neste estudo foi explorado em células deciduais de rato, não só o papel do metabolismo oxidativo da COX-2 na apoptose induzida pela AEA mas também os mecanismos moleculares subjacentes. Verificou-se que o metabolismo oxidativo da AEA estava envolvido nos efeitos pro-apoptóticos observado, e assim, as prostamidas podem ser consideradas novos mediadores do destino das células deciduais. Por outro lado, a COX-2 poderá desempenhar um papel chave na regulação dos níveis de AEA durante a remodelação decidual. Assim, uma desregulação na sinalização do ECS pode favorecer a produção de metabolitos oxidativos da AEA e, deste modo, interferir na estabilidade do tecido decidual.

O processo de decidualização é controlado por uma rede complexa de fatores de crescimento, citocinas, interleucinas, hormonas e outras moléculas, como os lípidos, nomeadamente os eCBs e as prostaglandinas (PGs), que parecem desempenhar um papel central neste processo. Contudo, os mecanismos celulares subjacentes ainda não estão totalmente identificados. Neste sentido, outro objectivo deste projeto foi estudar o envolvimento da AEA na proliferação, diferenciação e morte numa linha celular de células do endométrio e em culturas primárias de células deciduais fibroblásticas derivadas de

placentas de termo. Assim, o ECS foi caracterizado e foi identificado o papel da AEA, como inibidor da proliferação celular e do processo de diferenciação. O efeito anti-proliferativo da AEA foi observado nas células não diferenciadas e em células diferenciadas e foi associado ao aparecimento de poliploidia. Estes eventos celulares foram independentes da acção do CB1, mas parecem estar relacionados com um efeito direto da AEA no processo de divisão citoplasmático. Pelo contrário, e efeito inibitório da AEA no processo de diferenciação foi dependente da activação do CB1.

Tal como os eCBs, as PGs podem ser utilizadas como biomarcadores da recetividade uterina e implantação. No entanto, devido às dificuldades de quantificação das PGs *in vivo* ainda não foi possível a sua aplicação na clínica. Para superar este obstáculo, foi desenvolvido e validado um método de espectometria de massa de alta resolução para quantificar a PGE_2 e a $PGF_{2\alpha}$ em meio de cultura celulares. Recorrendo a esta metodologia o *cross-talk* entre os eCBs e as PGs durante o processo de diferenciação *in vitro* foi investigado. Além disso, a AEA foi proposta como um mediador do processo de decidualização, através da inibição da expressão da COX-2 e da produção de PGE_2. Estes efeitos foram mediados pelo recetor CB1. Curiosamente, também se verificou-se uma correlação inversa entre os níveis de AEA e PGE_2, a qual poderá ser um factor importante neste processo. No seu conjunto, estes resultados sublinham ainda que os eCBs e as PGs podem ser utilizados como potenciais biomarcadores da (in)fertilidade.

A anandamida controla o destino celular de uma forma específica tendo em conta o estadio de desenvolvimento do endométrio, o contexto ambiental e o tipo celular. Desta forma, o impacto da desregulação das vias de sinalização da AEA nas células endometriais do estroma uterino foi estudado. Pela primeira vez, observou-se que a ocorrência de apoptose em células não diferenciadas estava associada ao metabolismo oxidativo da AEA pela COX-2. Este fato enfatiza a importância dos níveis altamente regulados da AEA na proliferação das células do endométrio um processo crucial para a diferenciação.

Em conclusão, este trabalho contribuiu para a compreensão da base molecular que regula o *turnover* celular do endométrio materno e do processo de diferenciação. Alterações nas vias de sinalização da AEA e/ou do cross-talk entre a AEA e a PGE_2 durante a decidualização podem prejudicar os processos celulares envolvidos na remodelação endometrial que, em última análise, podem interferir com a resposta decidual, a gravidez e levar à infertilidade.

Palavras-chave: sistema endocanabinóide, anandamida, endométrio, decidualização, apoptose.

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Δψm - mitochondrial membrane potential
12-,15-HETE-EA - 12-,15-hydroxyeicosatetraenoic acid ethanolamide
12-,15-HETE-GE - 12-,15-hydroxyeicosatetraenoic acid glycerol ester
12-,15-HPETE-EA - 12-,15-hydroperoxyeicosatetraenoic acid ethanolamide
12-,15-HPETE-GE - 12-,15-hydroperoxyeicosatetraenoic acid glycerol ester
2-AG - 2-arachidonoylglycerol
2-AGE - 2-arachidonoyl glyceryl ether
3-β-HSD - 3-β-hydroxysteroid dehydrogenase
AA - arachidonic acid
ABHD4 - α/β-hydrolase 4
ABHD6 - α/β-hydrolase 6
AC - adenylyl cyclase
AEA - *N*-arachidonoylethanolamine
AIBP - anandamide intracellular binding proteins
AMD - antimesometrial decidua
ART - assisted reproductive technologies
ATP - adenosine triphosphate
C/EBP1 - CCCAT/enhancer-binding protein
cAMP - cyclic AMP
BMP - bone morphogenetic protein
CB - cannabinoid
CB1 - cannabinoid receptor 1
CB2 - cannabinoid receptor 2
CBD - cannabidiol
CNS - central nervous system
COX-2 - cyclooxygenase-2
cPLA2 - cytosolic phospholipase A-2
CPS - capsaicin
CRE - cAMP response element
CREB - cAMP response element binding protein
CRH - corticotrophin releasing hormone
CSF - colony-stimulating factor
CYP450 - cytochrome P450
DAG - 1,2-diacylglycerol
DAGL - diacylglycerol lipase
DKK1 - dickkopf-1
DSCs - decidualizing stromal cells
EA - ethanolamine

eCB - endocannabinoid

ECM - extracellular matrix

ECS - endocannabinoid system

EGF - epidermal growth factor

EMT - endocannabinoid membrane transporter

Epac - exchange protein directly activated by cAMP

ER - endoplasmic-reticulum

ERK - extracellular signal-regulated kinase

FAAH1 - fatty acid amide hydrolase 1

FAAH2 - fatty acid amide hydrolase 2

FABP - fatty acid binding protein

FAK - focal adhesion kinase

FAN - factor associated with neutral sphingomyelinase activation

FLAT - FAAH-like anandamide transporter

FOX01 - forkhead box 01

FSH - follicle stimulating hormone

GASP1 - G-protein receptor-associated sorting protein

GDE1 - glycerophosphodiesterase 1

GM-CSF - granulocyte-macrophage colony-stimulating factor

GnRH - gonadotrophin-releasing hormone

GPCR - G-protein-coupled receptor

GP-NAE - glycerophospho-*N*-acylethanolamine

GPR55 - G-protein-coupled receptor 55

hCG - human chorionic gonadotrophin

HdF - human decidual fibroblasts

HESCs - human endometrial stromal cells

HLA - human leucocyte antigen

HoxA - hox gene

HsP - heat shock protein

ICM - inner cell mass

IGFBP-1 - insulin growth factor-binding protein 1

IUGR - intrauterine growth restriction

IVF - *in vitro* fertilization

JNK - c-jun *N*-terminal kinase

Kir - inwardly rectifying potassium channels

LH - luteinizing hormone

LIF - leukemia inhibitory factor

LC-MS - liquid chromatography-mass spectrometry

LOX - lipoxygenase

LPA - lysophosphatidic acid

LPS - lipopolysaccharide

MAG - monoacylglycerol

MAGL - monoacylglycerol lipase

MAPK - mitogen-activated protein kinase

MD - mesometrial decidua

MMP - matrix metalloproteinase

NAAA - *N*-acylethanolamine-hydrolyzing acid amidase

NADA - *N*-arachidonoyldopamine

NAE - *N*-acylethanolamine

NAGly - *N*-arachidonoylglycine

NAPE - *N*-arachidonoyl-phosphatidylethanolamine

NAPE-PLD - *N*-acylphosphatidylethanolamine-specific phospholipase D

NAT - *N*-acyltransferase

ODA - oleamide

OEA - *N*-oleoylethanolamine

PA - phosphatidic acid

pAEA - phosphoanandamide

PARP-1 - poly (ADP-ribose) polymerase 1

PC - phosphatidylcholine

PEA - *N*-palmitoylethanolamine

PE - phosphatidylethanolamine

PG - prostaglandin

PGE_2 - prostaglandin E2

$PGF_{2\alpha}$ - prostaglandin F2 α

PG-EA - prostaglandin-ethanolamide

PG-GE - prostaglandin-glycerol ester

PGH-EA - endoperoxide prostaglandin ethanolamide

PI - phosphatidylinositol

PI3K - phosphoinositide 3-kinase

PKA - protein kinase A

PKB - protein kinase B

PLA1 - phospholipase A1

PLA2 - phospholipase A2

PLC - phospholipase C

PM - prostamide

PME_2 - prostamide E2

$PMF_{2\alpha}$ - prostamide F2α

PPAR - peroxisome proliferator-activated receptor

PRL - prolactin

PTPN22 - protein tyrosine phosphatase non-receptor type 22

PTX - pertussis toxin

ROS - reactive species of oxygen

SA - spiral artery

SM - sphingomyelin

SMase - sphingomyelinase

SPM - palmitoyltransferase

sPLA2 - phospholipase A2

St-T1b - telomerase-immortalized human endometrial stromal cell line

TE - trophectoderm cells

TGF - transforming growth factor

THC - Δ9-tetrahydrocannabinol

TNF - tumor necrosis factor

TRAIL - TNF-related apoptosis-inducing ligand

TRPV1 - transient receptor potential vanilloid 1

uNK - uterine natural killer

UPLC-MS/MS - ultra-performance liquid chromatography-mass spectrometry

VEGF - vascular endothelial growth factor

WNT - Wingless

WOI - window of implantation

CHAPTER I

Introduction

1. Milestones of (Endo)Cannabinoids history: a lesson to learn from *Cannabis Sativa*

Marijuana from the hemp *Cannab*is *sativa*, and its various alter egos, such as bhang and hashish are among the most popular illegal drug used worldwide. For millennia, *C. sativa* has been used medically, recreationally, and spiritually throughout the world. The earliest anthropological evidence comes from the oldest known Neolithic culture. In many ancient cultures (e.g., Chinese, Indian, and Tibetan), it was used to treat an endless variety of disorders including gastrointestinal disturbances, malaria, pain, snake bites, rheumatism and disorders of the female reproductive system (1).

It contains at least 400 chemical components of which over 60 are cannabinoids (2). The identification of cannabidiol (CBD) in 1963, and shortly after of Δ9-tetrahydrocannabinol (Δ9-THC; THC) constitute the major breakthroughs in the milestones of the (endo)cannabinoids history (3). The cannabinoid THC is the most psychoactive component of the plant. Due to its strong hydrophobic nature, it was firstly believed to exhibit nonspecific interactions with cell membranes (4). By the mid 1980's, the synthesis of THC enantiomers and the finding that cannabinoid activity was highly stereoselective prompted the beginning of the endocannabinoid system (ECS) research, an ensemble of the cannabinoid receptors (CB), their endogenous ligands, known as endocannabinoids (eCB), the membrane transporter and the enzymes responsible for the metabolism of eCBs (5).

Over the last decades, the recognized role of ECS in a myriad of physiological and pathological processes has opened new strategies based on their pharmacological properties (6). We currently stand at an interesting crossroads for *Cannabis*-related drugs, however, the addictive and psychomimetic properties considerably hamper the translation of the potential medical benefits. Nowadays, the therapeutic areas best associated with *Cannabis*-related medicines include chemotherapy-induced nausea and vomiting, nociception, feeding disorders, glaucoma, neurodegeneration/neuroprotection, multiple sclerosis, emotional disturbances, such as, anxiety and other psychiatric disorders including drug addiction, schizophrenia and epilepsy, as well as asthma and inflammatory disorders (7).

The ECS offers a great opportunity of therapeutic based-drug targets, such as agonists or antagonists of CB receptors and regulators of eCBs metabolic enzymes. In fact, there are already medicines that activate cannabinoid receptors available in the clinic, such as the synthetic analogues of THC, Cesamet® (nabilone), Marinol® (dronabinol; Δ^9-tetrahydrocannabinol) and Sativex® (Δ^9-THC/cannabidiol). The first two,

are prescribed to reduce chemotherapy-induced nausea and vomiting. Marinol® can also be used to stimulate appetite, while Sativex® was approved for the symptomatic relief of neuropathic pain in multiple sclerosis and as an adjuvant analgesic for advanced cancer (8). The selective cannabinoid receptor 1 (CB1) antagonist, Rimonabant (Acomplia®), was effective for the management of obesity, though the therapeutic doses increased the risk of psychiatric adverse effects, such as depression, anxiety and suicide. It was withdrawn from the European market in 2009 (9). The neuroprotective role of eCBs and their action on neurodegenerative diseases, such as Alzheimer's disease, Parkinson's disease, multiple sclerosis and Huntington's disease are also emergent focus of research (10). Nevertheless, as it will be discussed later, CB receptors have more than one endogenous ligand and, on the other hand, (endo)cannabinoids may activate several other receptors with different efficacy and affinity, ranging from ion channels to nuclear receptors. Therefore time will tell whether the range of realistic therapeutic areas for *Cannabis*-related medicinal drugs is as broad as the promise.

2. The Endocannabinoid System

2.1 Cannabinoid receptors

Cannabinoid receptors are members of the superfamily of G protein-coupled receptors (GPCRs) localized on the cell membrane with an extracellular binding site domain. More than two decades after THC discovery, Allyn Howlett found that cannabimimetic drugs induced inhibition of adenyl cyclase (AC) and decreased cyclic adenosine monophosphate (cAMP) levels in neuronal cells by a receptor-mediated mechanism (11). Subsequently, the first selective binding site for THC, the cannabinoid receptor 1 (CB1), was identified in 1988 and cloned from rat brain a year later (12, 13). Then, in 1993, the second cannabinoid receptor (CB2) was cloned from human promyelocytic leukaemia HL-60 cells (14). The receptors CB1 and CB2 share only 44% of homology, but their binding domains exhibit an identity of 69%, and, thus, most ligands do not discriminate between them (14, 15). CB1 receptor has been conserved throughout evolution. It has 97-99% amino acid sequence identity across human, rat and mouse species (13), whereas the CB2 sequence of rat and human present only 81% of identity (16). CB1 receptor is primarily expressed in the central nervous system (CNS), mainly in neocortical and hippocampal areas, but also in cerebellum and basal ganglia. This pattern of expression correlates with the central effects of THC, i.e. memory impairment, catalepsy, analgesia, hypothermia and immobility (17). Besides, it is also expressed in a

more widespread fashion in the peripheral system, such as fat, spleen, tonsils, skeletal muscle, small intestine, liver and reproductive tissues (18, 19). On the contrary, CB2 receptor is abundantly expressed in the immune system, however, it was also found in the CNS, in a more restricted distribution than CB1 and increased upon certain pathological conditions, such as neurodegenerative disorders (14, 19).

Accumulating evidences have shown that CB1, but not CB2, can be found in the lipid rafts, which are membrane microdomains enriched in cholesterol, sphingolipids and arachidonic acid that can modulate receptor activity and limit signal transduction (20). In fact, it is conceivable that changes in membrane fluidity/rigidity may affect ligand recognition and binding properties of CB1 (21). For instance, the reduced membrane fluidity resulting from cholesterol enrichment, reduced AEA-binding to CB1 receptor and enhanced membrane transporter activity (22). On the other hand, lipid rafts may also represent a cellular device of CB1 intracellular trafficking by constitutive endocytosis and degradation in lysosomes (23, 24).

Over the last years, orphan GPCRs have emerged as candidates for putative CB receptors. For instance, GPR55 was firstly considered the third CB receptor (CB3) (25). Although, besides eCBs, many other lipid-derived ligands are able to activate this receptor, which together with low homology with CB receptors, challenge its recognition as a cannabinoid receptor (26-28).

2.2 Endocannabinoids

By definition, endocannabinoids (eCBs) are endogenous compounds capable of binding to and functionally activate cannabinoid receptors, mimicking the cellular effects of THC. Chemically, these compounds are amides, esters or ethers of long-chain polyunsaturated fatty acids and are structurally different from THC but share critical pharmacophores with it (29). The molecular structure of THC and eCBs are represented in Figure 1.

N-arachidonoylethanolamine (Anandamide; AEA) was the first eCB identified, followed by 2-arachidonoylglycerol (2-AG). These are the best characterized members of the eCBs family, N-acylethanolamines (NAE) and monoacylglycerols (MAG), respectively (30, 31). They are present in the CNS, plasma and peripheral tissues (31, 32). Although eCBs share molecular structures and physical properties, their affinity to CB receptors varies. Anandamide is a CB1 partial agonist, whereas 2-AG is a CB1/CB2 full agonist.

Other eCBs were identified during the last years (Fig. 1), including 2-arachidonyl-glyceryl ether (noladin ether, 2-AGE) (33), O-arachidonoylethanolamine (virodhamine)

(34), N-arachidonoyldopamine (NADA) (35), N-arachidonoylglycine (NAGly) (36), and Cis-9,10-octadecanoamide (oleamide or ODA) (37). Their physiological significance, however, is not yet clarified. N-oleoylethanolamine (OEA) and N-palmitoylethanolamine (PEA) are considered endocannabinoid-like molecules whose effects may partially be attributed to their interference with enzymes involved in the eCB metabolism, but devoid of CB receptor activity (38).

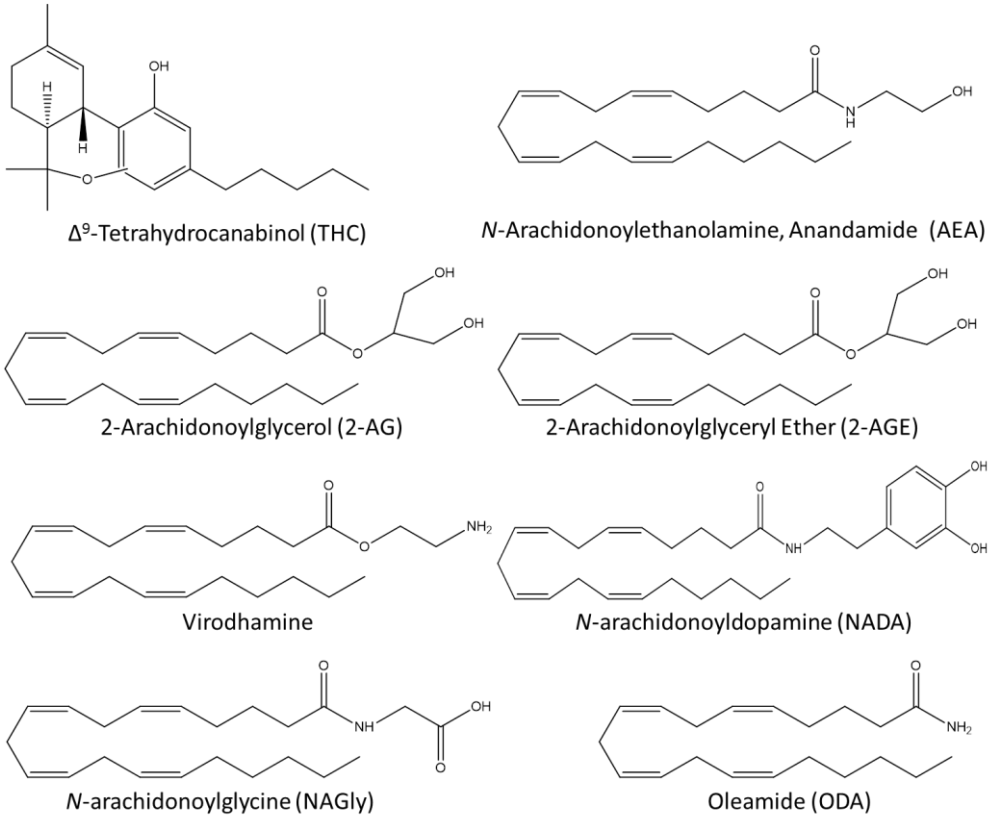

Figure 1. Chemical structures of the psychoactive phytocannabinoid THC and of the endogenous cannabinoids.

2.2.1 *N*-arachidonoylethanolamine (AEA)

Anandamide (*N*-arachidonoylethanolamine, AEA) was the first endogenous cannabinoid discovered in 1992 in the porcine brain (39). The name derives from the Sanskrit word *ananda*, which means “bliss, delight” and amide derived from its chemical structure. Tissue levels of AEA are very low, apart from the uterus and human brain, and are maintained by its metabolic enzymes (40, 41). The main biosynthetic enzyme of AEA is N-acylphosphatidylethanolamine (NAPE-PLD) and the fatty acid amide hydrolase (FAAH) is the enzyme responsible for its hydrolysis (42). Anandamide is a high affinity

partial agonist for CB1 receptor, an unusual feature for an endogenous ligand, while, like THC, it exhibits lower affinity for CB2 (43).

Anandamide is a relevant modulator in several physiological events, not only in the CNS, as a retrograde messenger, but also in the peripheral system (44, 45). For instance, AEA has antinociceptive effects (46, 47), induces bone proliferation and differentiation (48, 49), controls appetite and energy balance (50, 51) and mediates anti-inflammatory immune responses (52, 53). In the reproductive biology, it modulates a wide spectrum of processes from folliculogenesis, ovulation, implantation, placental development to spermatogenesis and sperm function (54-58). Moreover, AEA also regulates basic cellular processes, such as cell differentiation (49, 59-62), cell survival and death (63-67), and also autophagy (68), with different outcomes depending on the molecular targets activated and the cellular context.

While THC binds exclusively at CB receptors, eCBs exhibit a higher degree of "promiscuity" in terms of binding sites. The best established non-cannabinoid receptor for AEA is the transient receptor potential vanilloid type-1 (TRPV1) channel (69). This non-selective cation channel belongs to six-trans-membrane-domain transient receptor potential channels. It was first identified as the receptor for the pungent active principle of hot chilli peppers, capsaicin, however, it is also activated by plant toxins, noxious temperatures, low pH and intracellular redox states (70). TRPV1 mediates some AEA-induced effects, such as vasodilatation and cell apoptosis (69, 71-73). In addition, AEA is also capable to activate the peroxisome proliferator-activated receptor (PPAR) family of nuclear receptors, which may modulate AEA immune responses, neuroprotection, feeding behaviour and analgesia (74, 75). These receptors are localized intracellularly, in contrast to the extracellular domain of CB receptors' binding site. Moreover, there is growing evidence for the presence of non-receptor mediated-effects of AEA, such as the anti-inflammatory and proapoptotic actions through NF-κB inhibitory activity (76).

2.2.2 *N*-arachidonoylglycerol *(2-AG)*

2-Arachidonoylglycerol (2-AG) is the second "major" endogenous cannabinoid. It was identified in 1995 by Mechoulam *et al.* in the canine gut (32). Unlike AEA, 2-AG is a full agonist and binds equally to both receptors (77, 78). In addition, 2-AG may also activate PPAR-γ, although it does not bind TRPV1 (79). In rodents' uterus, endogenous 2-AG levels are generally about 200-fold higher than AEA (80) and almost 800-fold higher in brain (31). Hence, it is considered one of the principal eCBs involved in the protection in neurodegenerative processes (81-83), but it is also involved in other physiological

processes, including cell survival, differentiation and death (84-87). Moreover, 2-AG appears to be the true endogenous ligand for the peripheral CB2, controlling analgesia, food intake, immune responses and reproductive events (32, 88-92).

2.3 Biochemistry of the endocannabinoid system

Endocannabinoid levels are finely controlled by its metabolic enzymatic machinery. In fact, in pathological processes, eCB signalling might become enhanced or decreased, either to restore homeostasis or contribute to its symptoms and progression. By virtue, eCBs are present in trace levels in tissues and cells, and their synthesis from cell membrane phospholipid precursors is stimulated *on demand* through diverse metabolic pathways (Figure 2). They act as autocrine and paracrine mediators and modulate basic biological processes including cell proliferation, differentiation and apoptosis (29).

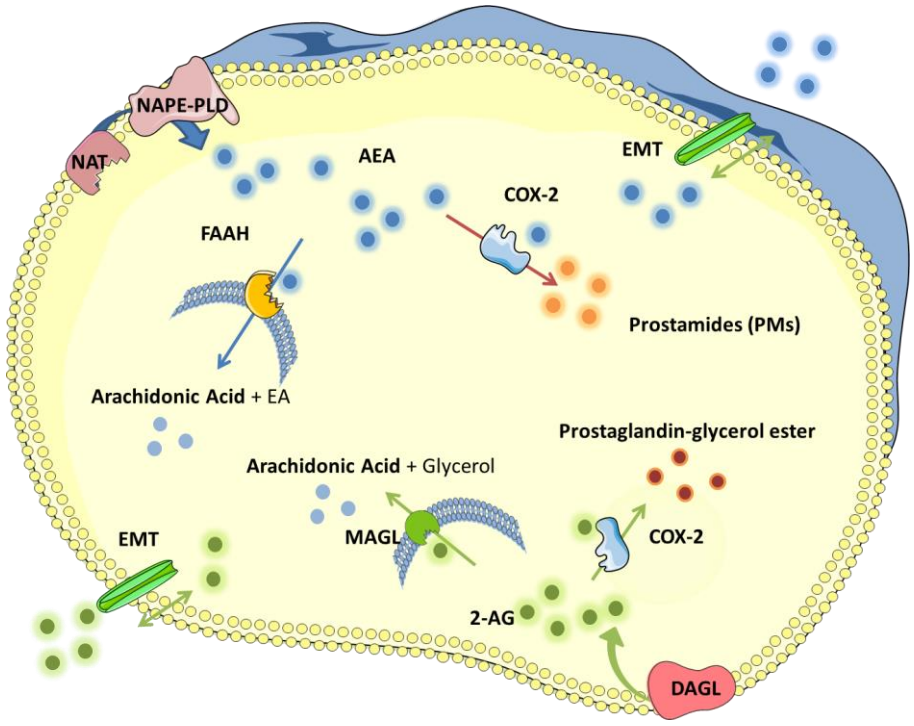

Figure 2. The endocannabinoid system. AEA (blue circles) biosynthesis from membrane precursors is catalysed by the enzyme NAT followed by NAPE-PLD. 2-AG (green circles) synthesis occurs from membrane precursors through DAGL enzyme. Once synthesized, eCBs are transported in both directions of the plasma membranes by simple diffusion or trough the transporter EMT. After cellular uptake they are rapidly degraded. AEA is hydrolysed by FAAH into EA and AA or by an alternative oxidative mechanism, by COX-2 into prostaglandin-ethanolamides or PMs (orange circles). 2-AG is mainly hydrolysed by the enzyme MAGL into glycerol and AA, and by COX-2 into prostaglandin-glycerol ester (pink circles). AA: arachidonic acid; AEA: anandamide; DAGL: diacylglycerol lipase; Cyclooxygenase-2: COX-2; EA: ethanolamine; EMT: endocannabinoid membrane transporter; FAAH: fatty acid amide hydrolase; MAGL: monoacylglycerol lipase; NAT: N-acyltransferase; NAPE-PLD: N-acyl-phosphatidylethanolamines-specific phospholipase D; PMs: prostamides; 2-AG: 2-Arachidonoylglycerol.

2.3.1 Biosynthesis of Anandamide

The synthesis of AEA occurs upon a stimulus, such as cell depolarization or mobilization of intracellular Ca^{2+} stores. The classic biosynthetic pathway of AEA is a two-step process (Figure 3). Firstly, the membrane Ca^{2+} dependent enzyme N-acyltransferase (NAT) synthesizes and releases *N*-acylphosphatidylethanolamine (NAPE) (93). NAPE is formed by the transfer of an arachidonate from sn-1 position of the membrane 1,2-diacylglycerolphospholipid to the amine group of phosphatidylethanolamine (94). Subsequently, AEA is released by NAPE-hydrolyzing-phospholipase-D (NAPE-PLD), a highly conserved member of the zinc metallo-lactamase-ß family Ca^{2+} dependent (95, 96). This enzyme uses as substrate, not only NAPE, the main precursor of AEA, but also other NAPEs with different N-acyl groups, generating their respective N-acylethanolamines (NAEs). Notably, NAPE accumulates in ischemic heart and brain or after toxic insults (97).

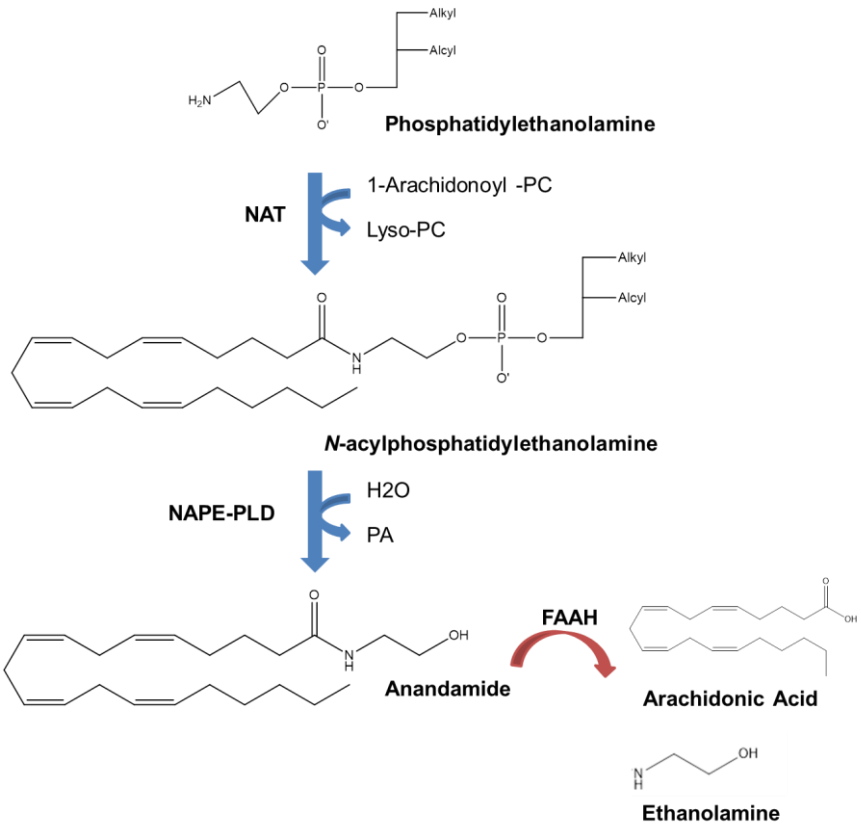

Figure 3. Major pathway for AEA synthesis and degradation. AEA synthesis involves the transfer of AA from 1-arachidonoyl-PC to PE by NAT, via the intermediate NAPE. The latter is then cleaved by NAPE-PLD releasing AEA and PA to the extracellular space. Anandamide is hydrolyzed by the enzyme FAAH into AA and EA. At blue and red are represented the principal biosynthetic and degradative pathways, respectively AA: arachidonic acid; AEA: anandamide; EA: ethanolamine; FAAH: fatty acid amide hydrolase; lyso-PC: lysophosphatidylcholine; NAT: N-acyltransferase; NAPE: N-acylphosphatidylethanolamine; NAPE-PLD: N-acyl-phosphatidylethanolamine- phospholipase D; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine.

Nonetheless, the finding of endogenous NAEs in NAPE-PLD knockout mice denoted the existence of alternative biosynthetic routes (98). In fact, NAEs may also result from NAPE through sequential deacylations, yielding lyso-NAPE and glycerophospho-ethanolamines. Accordingly, it has been described that phospholipase A2 (sPLA2) converts NAPE to 2-lyso-NAPE, which is then metabolized into AEA through a Ca^{2+}-independent mechanism by lyso-phospholipase D (lyso-PLD) (99). However, the restricted brain expression of sPLA2 suggested the involvement of additional enzymes in the production of lyso-NAPE (99). One such enzyme is the recently identified αβ-hydrolase 4 (Abhd4), which catalyses double deacylation of NAPE or directly interacts with lyso-NAPE to generate the glycerophospho-acylethanolamine (GP-NAE) (100). The latter, is then converted into NAEs by a metal-dependent phosphodiesterase, glycerophosphodiesterase 1 (GDE1) (101).

Another alternative pathway identified in the RAW264.7 mouse cells involve the hydrolysis of NAPE by a phospholipase C to yield phosphoanandamide (pAEA), which is then dephosphorylated by a tyrosine phosphatase, PTPN22, to form AEA (102).

2.3.2 Biosynthesis of 2-Arachidonoylglycerol

The metabolic pathway for the synthesis and degradation of 2-AG is displayed on Figure 4. In most cases, 2-AG is produced from the hydrolysis of membrane phospholipids through a phospholipase C (PLC), producing 1,2-diacylglycerol (DAG). The latter, is then converted to 2-AG by a diacylglycerol lipase (DAGL) (88). Regarding the enzymatic conversion of DAGs into 2-AG, two DAG lipase isozymes (DAGLα and DAGLß) have been cloned and enzymatically characterized (103). Alternatively, 2-AG biosynthesis may involve phospholipase A1 (PLA1), yielding lyso-phosphatidylinositol, which is then metabolized by lyso-phospholipase C (lyso-PLC). Much alike the enzymes involved in AEA biosynthesis, DAG lipases do not appear to be selective.

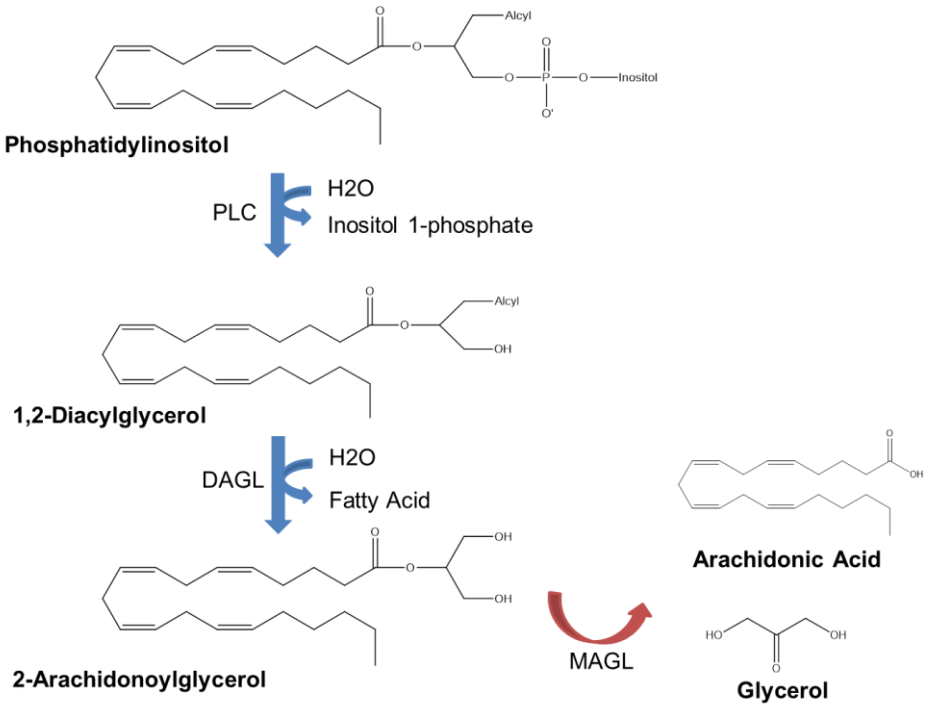

Figure 4. Major pathway for 2-AG synthesis and degradation. Firstly, membrane PI is cleaved by PLC yielding 1,2- DAG. The latter is dehydratated by DAGL releasing 2-AG. Following uptake, 2-AG is mainly hydrolyzed by MAGL, releasing AA and glycerol. FAAH may also hydrolyze 2-AG. At blue and red are represented the principal biosynthetic and degradative pathways, respectively. AA: arachidonic acid; DAG: diacylglycerol; DAGL: diacylglycerol lipase; MAGL: monoacylglycerol lipase; PLC: phospholipase C; 2-AG: 2-arachidonoylglycerol.

2.3.3 Cellular uptake and degradation of eCBs

Termination of eCB signalling occurs in a two-step process involving the transport into the intracellular space followed by rapid degradation. The accepted models proposed for eCBs uptake and intracellular trafficking will be discussed below and are resumed on Figure 5. Mostly, this knowledge relies on studies exploiting AEA, though it appears that 2-AG may also be transported and accumulated in cells by common mechanisms. Nevertheless, this research field is still at the present a matter of controversy. Due to their lipophilic nature, eCBs are believed to cross cell membranes by simple diffusion, in favour of the concentration gradient, which is essentially determined by the intracellular sequestration and hydrolysis (104). Additionally, it has been reported a facilitated diffusion mechanism through a specific and substrate selective transporter (105). For long, a still currently molecular-uncharacterized transporter, referred to as “endocannabinoid membrane transporter” (EMT), temperature sensitive, saturable, bidirectional and energy-independent has been described (106). Meanwhile, in 2011 Fu *et al*. found a variant of the enzyme FAAH-1 that acts as a specific transporter of AEA in neurons and other cell types.

This enzyme, designated FAAH-1–like anandamide transporter (FLAT), lacks amidase activity, but appears to facilitate AEA uptake (107). Nevertheless, it has also been recently proposed as an intracellular protein transporter with residual catalytic activity (108). Moreover, there is also evidence that AEA can be internalized by a caveola-related endocytic process (109).

Even less understood is the intracellular trafficking of eCBs (Figure. 5). Recent insights on intracellular stores and binding proteins challenged reconsideration on the dogma that eCBs are exclusively synthesized *on demand*. In fact, purported intracellular stores, named adipossomes, have been linked to trafficking and accumulation of AEA, maintaining a concentration gradient that enables simple diffusion. Moreover, since they have been associated with degrading enzymes, they may also be responsible for the metabolic control of AEA (110).

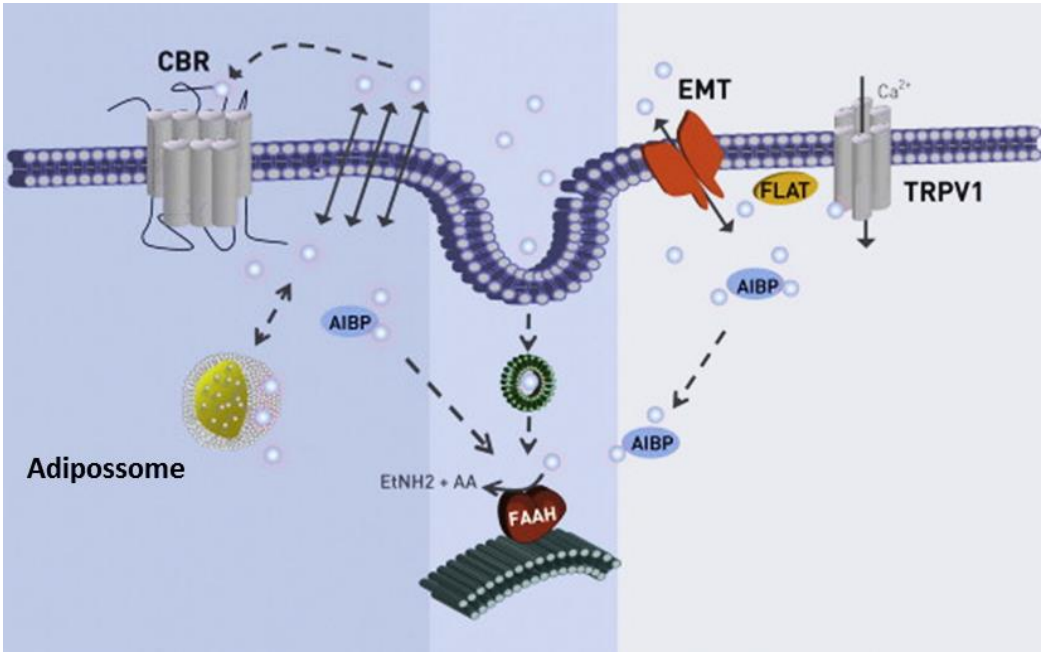

Figure 5. Major pathways for AEA uptake and intracellular trafficking. In the left (dark blue), AEA concentration gradient drives simple diffusion across the plasma membrane; in the center (blue), AEA reuptake occurs via a caveolae-related endocytic process; in the right (grey), AEA is transported across the plasma membrane by a carrier protein, EMT. The intracellular sequestration of eCBs by binding to intracellular proteins has also been suggested as a mechanism for eCBs uptake and to maintain the gradient concentration of AEA. Hsp70, FABP5 and FABP7 are reported to be AIBP. AEA can be stored in adiposomes that can also interact with AIBP. Additionally, FLAT facilitates the internalization and transport of AEA on the cell. AEA: anandamide; Anandamide Intracellular Binding Proteins: AIBP; CBR: cannabinoid receptor; EMT: endocannabinoid membrane transporter; FAAH: fatty acid amyde hydrolase; FLAT: FAAH-1–like anandamide transporter; TRPV1: transient receptor potential vanilloid-1. Adapted from (42).

In addition, several intracellular transporters, Anandamide Intracellular Binding Proteins (AIBP) have been identified to facilitate the trafficking of AEA from the plasma membrane to different intracellular compartments, including intracellular receptors and/or degrading enzymes. Those currently established are fatty acid binding proteins 5 and 7 (FABP5/7), heat shock protein 70 (Hsp70), albumin, and FLAT (107, 108, 111). In the extracellular space, AEA transport may depend on carriers like serum albumin and lipid-binding proteins, such as lipocalins (112).

Following membrane uptake, AEA is primarily degraded by the specific fatty acid amide hydrolase (FAAH, or FAAH-1), yielding arachidonic acid (AA) and ethanolamine (EA) (113). FAAH is an intracellular membrane-bound enzyme member of the amidase signature family of enzymes. It is widely distributed throughout the CNS, mostly coincident to CB1 receptor localization, but it is also found at the periphery (114). FAAH has an alkaline optimal pH and preferentially hydrolyses AEA over other NAEs.

Contrary to rats, an isozyme of FAAH, FAAH-2 was discovered in humans, mainly in heart and ovaries (115). In contrast to the endoplasmic reticulum-localization of FAAH, FAAH-2 is expressed in lipid droplets, like adipossomes. The different distribution of the two FAAH enzymes might suggest different biological roles and FAAH-2 is also less efficacious than FAAH at hydrolysing NAEs (114, 116). A third NAE-hydrolysing enzyme was recently discovered, N-acylethanolamine-hydrolyzing acid amidase (NAAA), which appears to be highly expressed in immune cells, especially in macrophages, and localized in lysosomes (117). No sequence homology was found between NAAA and FAAH (118).

FAAH in some cases, may catalyse 2-AG hydrolysis to AA and glycerol (119). Nevertheless, monoacylglycerol lipase (MAGLs), present in both membrane and cytosolic subcellular fractions, is considered the main route of 2-AG enzymatic hydrolysis (Fig. 4) (119). MAGL may also recognize other unsaturated monoacylglycerols as substrates, which, in some cases, compete with 2-AG inactivation. Furthermore, unlike AEA, 2-AG can be re-esterified into phospholipids before being hydrolysed (88).

2.3.4 Oxidative metabolism of endocannabinoids

The oxidative metabolism represents an alternative route for termination of the eCBs-signalling. Nevertheless, it is also emerging as a metabolic pathway providing a wide range of novel oxidative-derivatives of endocannabinoids with physiological significance. As shown in Figure 6, AEA may be subjected to a direct oxidative metabolism by the same enzymes that are responsible for arachidonic acid oxidation. These include the cyclooxygenase-2 (COX-2), yielding prostaglandin-ethanolamides or prostamides (PMs),

the 12- and 15-Lipoxygenase (12-LOX, 15-LOX) which give rise to 12- and 15-hydroperoxyeicosatetraenoylethanolamide, 12- and 15-HETE-EA, respectively, and Cytochrome P-450 is able to oxidize AEA to hydroxyeicosatetraenoic and epoxyeicosatrienoic acid ethanolamides (120).

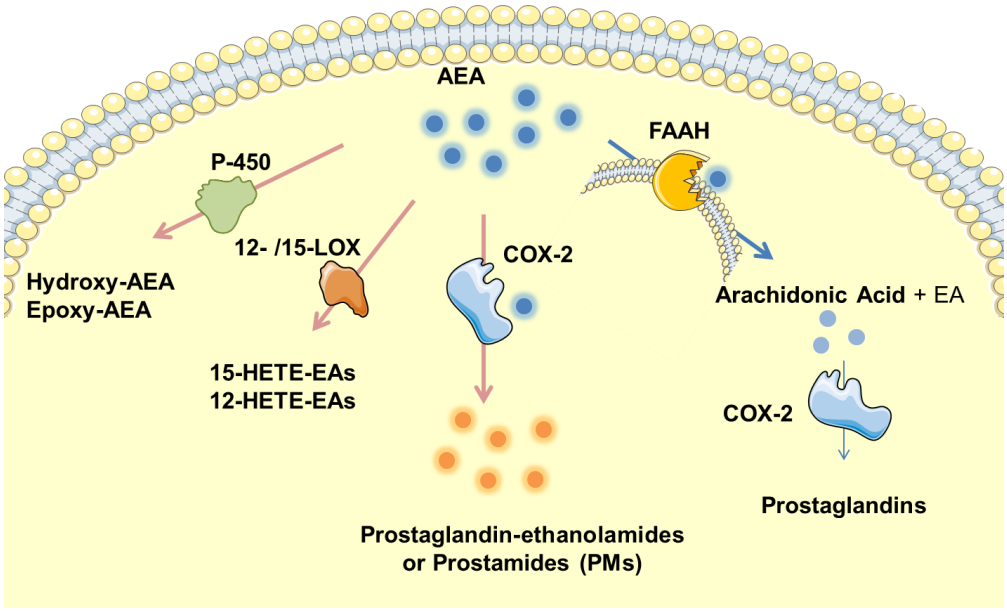

Figure 6. Metabolism of AEA. Oxidative metabolism of AEA by COX-2, the main oxidative pathway, 12- and 15-LOX and P-450, yielding PMs, HETE-EAs, and hydroxy- and epoxy-AEA, respectively. The hydrolytic metabolism by FAAH generates AA and EA. AA can be oxidized by COX-2 to form PGs. AA: arachidonic acid; AEA: anandamide; COX-2: cyclooxygenase-2; EA: ethanolamine; FAAH: fatty acid amide hydrolase; HETE-EAs: hydroxyeicosatetraenoic-ethanolamides; LOX: lipoxygenase; P450: cytochrome P450; PGs: Prostaglandins; PMs: prostamides.

As AEA, the other main eCB, 2-AG, was also described as substrate for COX-2, lipoxygenase, and cytochrome P-450 metabolism (121, 122). In contrast to AEA, COX-2 metabolizes 2-AG as efficiently as AA (121). COX-2 oxidative metabolism of 2-AG produces an identical repertoire of molecules as AEA, named prostaglandin-glyceryl esters (PG-GEs) (120, 121) (Figure 7). This mechanism involves an intermediate, the endoperoxide prostaglandin–glycerol ester (PGH2-glycerol ester), which is hydrolysed by yet unidentified enzymes to respective PG-GEs. Still little is known regarding the metabolic pathways of PG-GEs. Interestingly, it has been described that prostaglandin glycerol-ester D_2 (PGD_2-GE) is preferentially hydrolysed by the α/β-hydrolase enzyme ABHD6 (123), which also controls 2-AG activity at CB receptors (124). Moreover, PG-GEs were described as substrates for MAGL, the main hydrolytic enzyme of 2-AG (125). Different from prostamides, in rat plasma, PG-GEs are rapidly hydrolysed *in vivo* to the corresponding PGs (126, 127). 2-AG derived PG-GEs may play a role in physiological events (126, 128-130). For instance, the prostaglandin glycerol-ester E_2 (PGE_2-GE)

induced opposite effects to its precursors on inhibitory synaptic transmission and neurodegeneration, via CB1- and prostanoid- receptor-independent mechanisms (131-133).

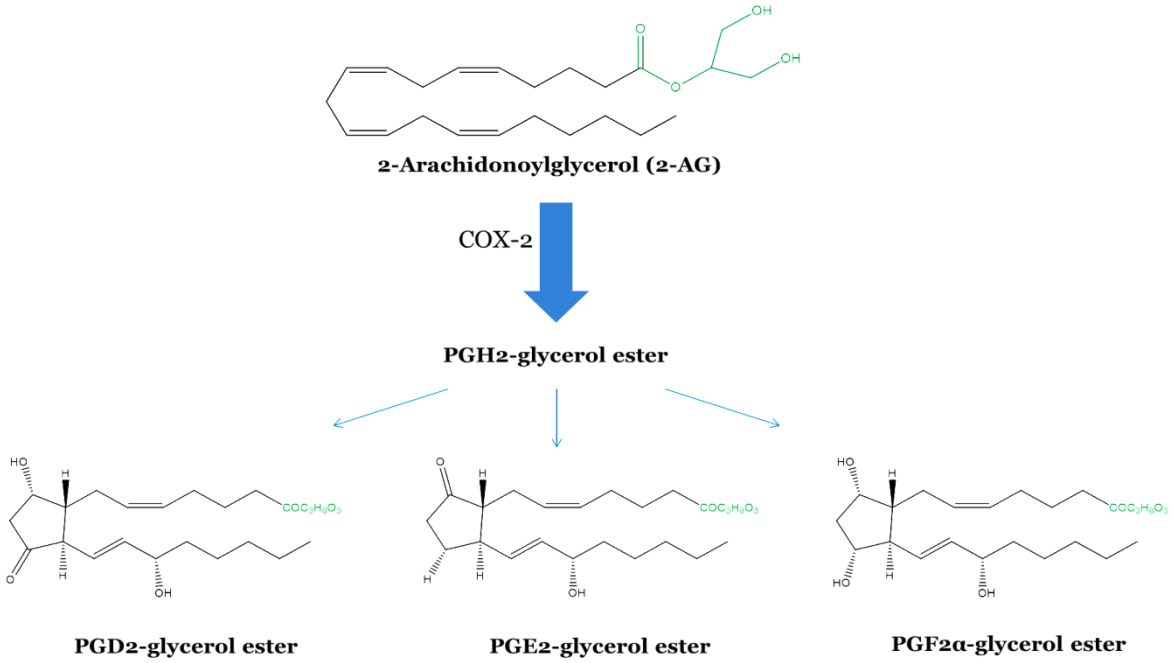

Figure 7. Oxidative metabolism of 2-arachidonoylglycerol by COX-2.

2.4 COX metabolism of anandamide and biological activities

Once inside the cells, AEA is rapidly hydrolysed by FAAH into arachidonic acid and ethanolamine. Cyclooxygenase-2 (COX-2) is an inducible enzyme that by using AA as substrate gives rise to prostaglandins (PGs). In fact, the latter have been associated with AEA-induced apoptotic and antiproliferative effects (134, 135), contraction or relaxation of isolated vascular ring preparations (136), changes in arterial pressure in perfused lung (137), adipocyte differentiation (138) and inhibition of IL-2 secretion in splenocytes (139).

On the other hand, the direct oxidative metabolism by COX-2 over AEA also constitutes one of the main alternatives routes of degradation, yielding prostamides. Although it has been reported by Yu *et al*. back in 1997 (140), the biological significance of PMs as lipid mediators is only currently emerging in the literature. Accordingly, AEA is a substrate of COX-2, but not of the constitutive COX-1, leading to the formation of the intermediate endoperoxide prostaglandin-ethanolamide (PGH2-EA). The latter is then converted in several prostamides, including PME_2, as a major product, $PMF_{2\alpha}$ and PMD_2, by tissue- and cell-specific PG synthases (120, 141, 142) (Fig. 8). With the exception of $PMF_{2\alpha}$ synthase, no other synthases for PMs have yet been identified (141, 143). The

former is abundantly expressed in male and female reproductive organs and also in the CNS (143, 144). Since low nanomolar concentrations of AEA are typically observed *in vivo* (145), it could be advocated that the production of such COX-2-derived metabolites was unlikely to occur *in vivo*, but this is starting to be demystified. Furthermore, it has been shown that under physiological conditions, in rat and human plasma and whole blood, PMs are highly stable to oxidation and hydrolysis, thus, having a longer half-life when compared to PGs and eCBs (142, 146). Regarding their metabolism, PMs are not substrates for 15-hydroxyprostaglandin dehydrogenase, the enzyme responsible for the initial step of PG inactivation (127) nor for FAAH (147-149), although, some reports described tissue-specific hydrolysis to the corresponding PGs (150, 151).

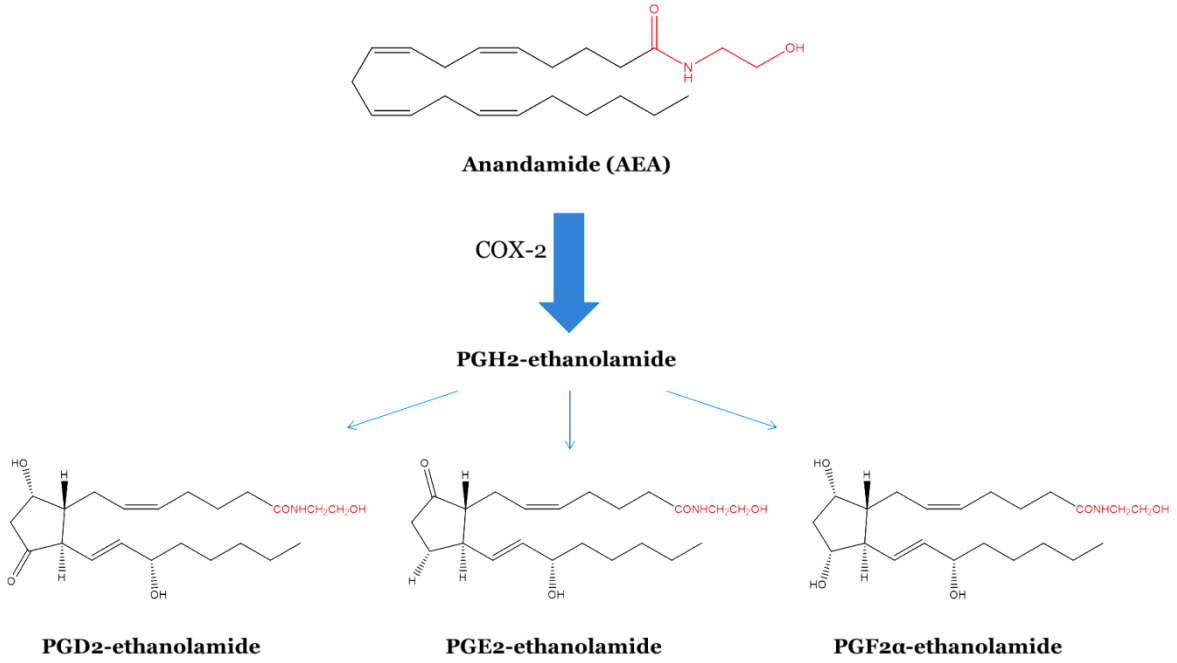

Figure 8. Oxidative metabolism of anandamide by COX-2.

Prostamides are not able to activate CB receptors (152) nor TRPV1 or PPAR (149), and exhibit no meaningful activity at PG receptors (147, 149, 150). Instead, several evidences point to a potential interaction with novel and yet-to-be characterized molecular targets (149). Hence, $PMF_{2\alpha}$, the most studied prostamide, appears to interact with a specific subset of receptors (152, 153). Also, they do not inhibit FAAH or MAGL by a feedback mechanism (148). The pharmacological properties of PMs are beginning to be disclosed, including in ocular hypotension and smooth muscle contraction (154-156). The first evidence of their biological effect came from the therapeutic efficacy of the $PMF_{2\alpha}$ analog, bimatoprost, used in the treatment of glaucoma (150, 157, 158). Moreover, the contractile activity of $PMF_{2\alpha}$ was described in rabbit uterus, feline iris and feline lung

parenchyma (149, 159, 160). In addition, the involvement of PMs in inflammatory conditions has been referred. In a rat-model of inflammatory pain, spinal $PMF_{2\alpha}$ levels were increased and shown to exert pro-inflammatory effects (161). PME_2, like AEA, was identified as an anti-inflammatory molecule in activated microglia cells, suppressing IL-12p40 expression (162). In addition, PME_2 reduced lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) production in human blood, monocytes and the THP-1 monocytic cell line (163). In a human explant colitis model, the AEA-induced anti-inflammatory effect was mediated by PME_2 and $PMF_{2\alpha}$ (164). Interestingly, in contrast to AEA-stimulatory adipogenic effect through CB1 and PPAR, $PMF_{2\alpha}$ is an antiadipogenic mediator (165, 166). The role of PMs in apoptosis has also been addressed. In fact, PMD_2 together with PME_2 mediated AEA-induced apoptosis in colorectal carcinoma cells, whereas, curiously, PGE_2 was shown to stimulate cell proliferation (151). Recently, a novel ethanolamide-conjugated J-series prostaglandin, (15d-PMJ2) was found to mediate selectively AEA-endoplasmic reticulum stress cell death in non-melanoma skin cancer that overexpresses COX-2 (167, 168).

Following the discovery that the anti-anti-inflammatory molecules like (R)-profens selectively inhibit eCB oxygenation over AA (169), a key role of COX-2 as a crucial regulator of the eCB tone has been established and the therapeutic exploitation of this metabolic route explored. In fact, (R)-flurbiprofen reduces neuropathic pain by restoring the endogenous eCB balance following neuronal injury (170). In this way, selective COX-2 inhibition has been proposed as novel therapeutic strategies for neuropathic pain, neuropsychiatric and anxiety disorders (171, 172).

In summary, COX-2 oxidative metabolism is more than a pathway to terminate eCB signalling and PMs are emerging as a novel class of eicosanoids with separate receptors and unique transduction pathways. On the other hand, as eCBs synthesis is activated following certain conditions, which often concur with COX-2 up-regulation, it is likely that eCB-derivatives may not only mediate or extend AEA-effects, but also exert their own activities. Moreover, PMs have been identified in much higher levels in several tissues from FAAH knockout mice (173), which are often characterized by elevated AEA levels.

2.5 Endocannabinoid signalling

Cannabinoid receptors are crucial to the transduction of extracellular stimuli into intracellular signals of endocannabinoids. The proposed signalling pathways triggered by eCBs through CB receptors are resumed in Figure 9. In contrast to other GPCR proteins, CB1 receptor is constitutively present with a high degree of basal activity, even in the

absence of exogenous agonists (174). Stimulation of CB1 receptor signal transduction, results in adenylyl cyclase (AC) inhibition (175), activation of the mitogen-activated protein kinase (MAPK) (176) and focal adhesion kinase (FAK) (177), activation of inwardly rectifying potassium (K_{ir}) channels and inhibition of voltage-gated Ca^{2+} channels (178). Moreover, CB1-inhibition of AC and decreased cyclic adenosine monophosphate (cAMP) accumulation consequently prevents protein kinase A (PKA) activation. These effects were blocked by pertussis toxin (PTX), suggesting the involvement of G inhibitory ($G_{i/0}$) proteins (179).

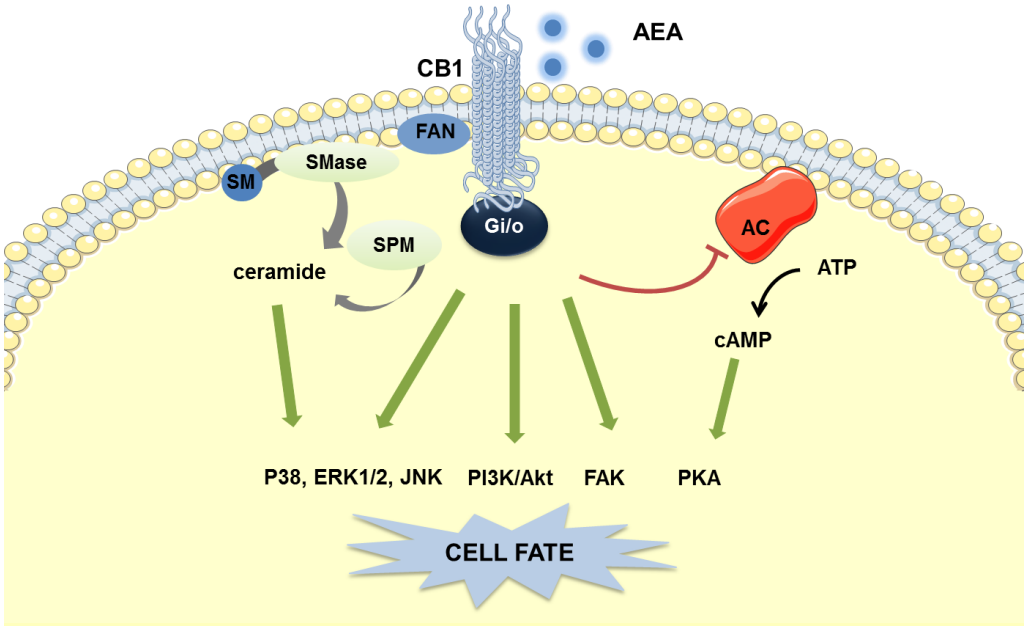

Figure 9. Main signal transduction pathways of CB1 activation. Coupled to $G_{i/0}$ proteins, CB1 activation by AEA (blue circles) leads to a manifold of signal transduction pathways including: inhibition of AC, decrease cAMP and PKA activity; activation of MAPK (ERK1/2, JNK, p38), PI3K/Akt and FAK kinases. Also, Ca^{2+} intracellular levels are increased. The binding of FAN protein to CB1 induces ceramide accumulation by sphingomyelin hydrolysis by SMase. *De novo* synthesis of ceramide is mediated by SPM. When CB1 triggers G_s protein, AC is stimulated increasing cAMP levels. AC: Adenylyl Cyclase; AEA: anandamide; ATP: Adenosine triphosphate; CB1: cannabinoid receptor 1; ERK1/2: Extracellular signal-regulated kinase 1/2; FAN: factor associated with neutral sphingomyelinase activation; FAK: focal adhesion kinase; JNK: Jun N-Terminal kinases; p38: p38 Mitogen-activated protein kinase; PKA: Protein Kinase A; SM: sphingomyelin; SMase: Sphingomyelinase; SPM: serinoylpalmitoyltransferase.

CB1 through $G_{i/0}$ stimulation, induces phosphorylation of MAPK kinase, including p42/p44 mitogen-activated MAPK also known as extracellular signal-regulated kinase 1/2 (ERK 1/2), Jun N-terminal kinase (JNK) and p-38, resulting in a manifold of cellular events such as cell differentiation, survival and death, cell migration, regulation of cytoskeletal status and intracellular trafficking (180-185). Recently, the neuroprotective role of CB1 receptor has been shown to involve phosphorylation of ERK1/2 and phosphoinositide 3-kinase/Akt (PI3K/Akt) pathway (186, 187). In fact, it is postulated that CB1 may enhance or inhibit PI3K/Akt pathway (188, 189). The inhibition of voltage-dependent ion channels,

primarily L-, N- and P/Q-type Ca^{2+} channels, and activation of K^+ channels is also dependent on CB1 activation coupled $G_{i/0}$ pathways (190-192). Although it is still controversial, CB1 may increase intracellular Ca^{2+} concentrations through different pathways (193, 194). CB2 does not seem to modulate ionic channels, but it is also able to increase Ca^{2+} levels (195).

Nevertheless, with lower efficacy, CB1 can also directly interact with G_s and $G_{q/11}$ proteins (196-198). Consistent with G_s activation, CB1 can stimulate cAMP production (199). The CB1-induced cAMP activation is dependent on the agonist-specific G-protein interactions, the isoform of AC expressed in target cells and also the way that each isoform responds to $G_{i/o}$-mediated regulation. As so, AC-I, III, V, VI and VIII are inhibited by CB agonists whereas AC-II, IV and VII are stimulated (200).

The knowledge that CB1 signal transduction can be synchronized via regulation of the second messenger ceramide unveiled a novel eCBs signalling pathway. The adaptor protein factor associated with neutral sphingomyelinase activation (FAN), mediates the interaction between CB1 and a neutral sphingomyelinase (SMase), which directs the hydrolysis of sphingomyelin (SM) leading to the transient production of ceramide. On the other hand, CB1 activation also induces a long term ceramide accumulation through stimulation of *de novo* synthesis through the serine palmitoyltransferase (SPM) (201).

2.5.1 Endocannabinoid controlling cell fate

As abovementioned, endocannabinoids differentially modulate cell proliferation, survival and differentiation in several (pato)physiological processes (Figure 10).

In human hepatocellular carcinoma cells, AEA inhibited cell proliferation by cellular arrest at the G1-S transition via upregulation of p21, and induced apoptosis trough upregulation of BCL2-antagonist/killer 1 (Bak) (202). In human breast and prostate cancer cells, AEA-induced cell cycle arrest at G1-S phase and apoptosis through CB receptors activation, cAMP/PKA inhibition and activation of ERK1/2 kinases (203-206). In addition, the inhibitory effect of eCBs on tumour cells proliferation is usually linked to a diminished activation of growth factors receptors, such as the epidermal growth factor receptor, the nerve growth factor receptor and the vascular endothelial growth factor receptor (VEGF-R), and to inhibition of downstream signalling pathways, such as the PI3K/Akt (207, 208). In rat C6 glioma cells, inhibition of cell proliferation involved activation of both CB and TRPV1 receptors (209), while in neuroblastoma cells it was through a lipid raft-dependent mechanism (210).

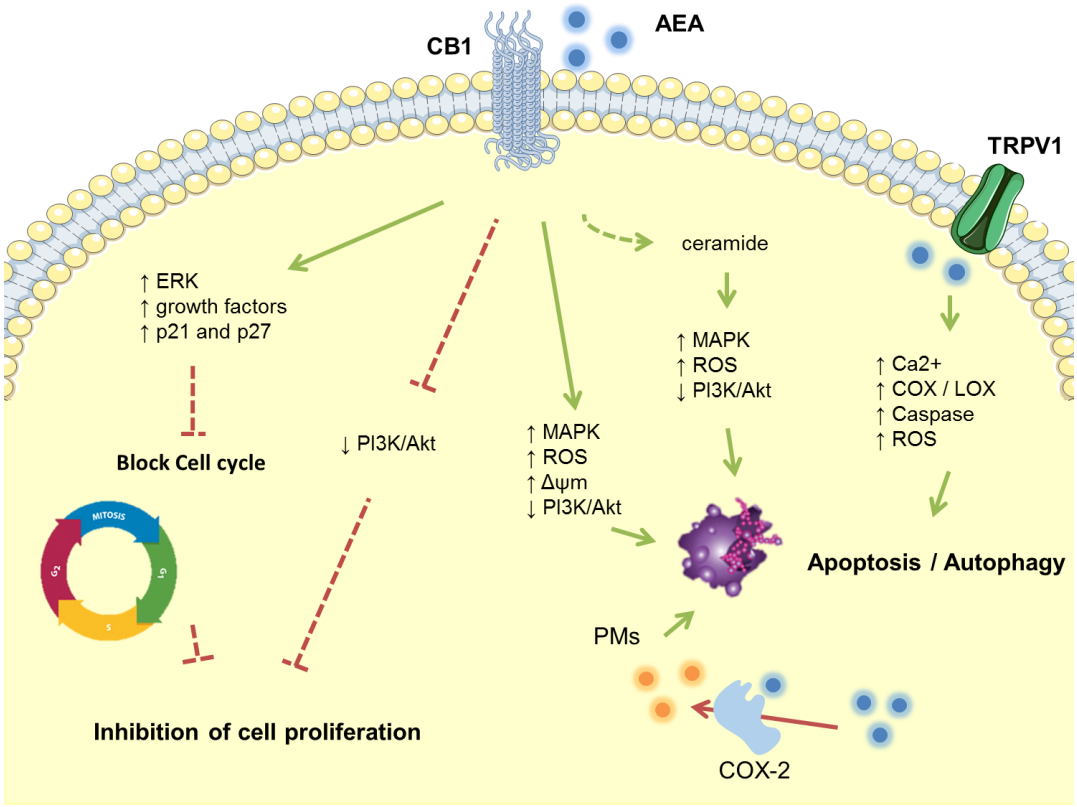

Figure 10. Control of cell fate by AEA. AEA (blue circles) blocks cell cycle progression through several pathways and inhibits activation of PI3K/Akt, a survival pathway. Apoptotic cell death induced by AEA may also derive from different cellular pathways, including increased production of ceramide and subsequently MAPK activation, ROS generation and inhibition of PI3K/Akt. In addition, binding of AEA to CB1 can trigger ROS production, activation of MAPK and induce mitochondrial transmembrane potential loss. Alternatively, AEA can activate TRPV1 triggering activation of caspases and the COX and LOX pathway as well as ROS prodction. On the other hand, PMs (orange circles) derived from oxidative metabolism of AEA may induce apoptosis. AEA: anandamide; Ca^{2+}: calcium; CB: cannabinoid receptor; COX: Cyclooxygenase; LOX: Lipogynease; MAPK: Mitogen-activated protein kinases; PKA: protein kinase A; PMs: prostamides; TRPV1: transient receptor potential vanilloid 1.

Anandamide is also able to induce apoptosis in PC-12 pheochromocytoma cells (211), CHP100 neuroblastoma cells (29), prostate cells (206) and uterine cervix cancer cells (212). In the reproductive biology field, AEA mediates cell death in human cytotrophoblast cells and in rat primary decidual cell cultures through a CB-receptor dependent mechanism (56, 65, 213). Most of the apoptotic cellular events abovementioned involve CB1-mediated phosphorylation of MAPK kinases, which may also be associated with oxidative stress, endoplasmic-reticulum stress, loss of mitochondrial transmembrane potential (Δψm), increased Ca^{2+} production and cytochrome *c* release (67, 213-215). However, in PC12 cells, AEA triggered apoptosis through direct activation of the apoptosis signal-regulating kinase 1 (ASK1) and p38/JNK pathways (216). Nevertheless, eCBs may also activate the death receptor pathway (86, 217) or both the intrinsic and extrinsic pathways (56, 67). In addition, as abovementioned,

activation of both CB receptors may trigger *de novo* synthesis of ceramide. The latter may also regulate apoptosis through activation of MAPK kinases and induced ROS production (185, 218-220). Interestingly, while ceramide elicits proapoptotic effects in glioma cells, CB1-induced ceramide act as a neuroprotector in astrocytes and neurons. These opposite effects were related to the PI3K/Akt pathway, which is inhibited in glioma but upregulated in glia by eCBs (221).

Anandamide-mediated production of cellular reactive oxygen species (ROS) also takes part in the apoptotic-signal transduction pathway. In this context, it must be recalled that LOX and COX, are redox stress sensors implicated in various death programs via production of AEA hydroperoxides. In PC12 cell line, in human coronary artery endothelial cells and primary hepatocytes, activation of CB1 by AEA resulted in apoptosis by generation of intracellular superoxide anion and altered redox state (211, 222, 223).

AEA also exhibits the ability to activate the TRPV1 receptor. In human endothelial cells, AEA-induced cell death through TRPV1-mediated phosphorylation of p38/JNK kinases (224). In neuroblastoma and lymphoma cells, AEA proapoptotic effect by TRPV1 binding, involved a series of events such as an increase in Ca^{2+}intracellular levels, activation of the COX and LOX pathways, release of cytochrome *c* and activation of caspases -9 and -3 (29, 225). On the contrary, in these cells, activation of CB1 promoted AEA uptake and subsequent hydrolysis thus preventing cell death. These observations suggested that the different localization of AEA-binding receptors, intracellular for TRPV1 and extracellular for CB receptors, may play a role in discriminating AEA-effects on cell fate (225). Furthermore, whereas high concentrations of AEA are associated with cell death in human melanocytes through a TRPV1-mediated pathway, lower AEA concentrations stimulate melanin synthesis (226).

Increasing evidences also point towards the role of lipid rafts in mediating eCB-induced apoptosis through a CB1-dependent mechanism (217, 227). In human neuroglioma cells a stable analogue of AEA induced apoptosis through lipid rafts, involving ceramide production, activation of MAPK and increased PGs synthesis (228). Furthermore, methyl-β-cyclodextrin (MCD), a membrane cholesterol depletor prevented AEA-apoptotic effects on hepatocytes in a mechanism independent of receptors (229).

Nevertheless, the frame picture is more complex, since it has been shown that eCBs are also able to modulate the PI3K/Akt, a pivotal antiapoptotic pathway (29). This finding is of particular interest, because it indicates a protective role of CB receptors against programmed cell death in human astrocytoma cells (230). However, inhibition of the PI3K/Akt pathway has also been reported in AEA-apoptotic role in cancer cells (207).

Additionally, proliferative properties of cannabinoids have been described in a manner dependent on CB2 activation (49, 231, 232).

Remarkably, eCBs have also ability to induce other types of cell death (227, 233). In fact, in rat primary decidual cell cultures, high AEA concentrations caused a dramatic cytotoxic effect in cell viability, which was preserved by depletion of the membrane cholesterol (65). On the other hand, cannabinoids have also been associated with pancreatic adenocarcinoma cell growth inhibition by a ROS-dependent autophagic cell death (234). Autophagy has also been described as a mechanism by which cannabinoids promote the activation of mitochondrial pro-apoptotic pathway, through inhibition of PI3k/Akt, induction of endoplasmic-reticulum stress and ROS generation, through CB2 in glioma cells and independently of CB receptors in breast cancer (235, 236).

Noteworthy, AEA also affects cell differentiation in glioma and astroglial cells (237, 238). In this regard, in neuronal cells, AEA inhibits cell differentiation through binding to CB1 receptor and attenuation of ERK1/2 kinases (62), and prevents keratinocyte differentiation by inducing DNA methylation (59). In reproductive systems, AEA limits rat decidual cell differentiation (239), while 2-AG impairs human cytotrophoblasts cells syncytialization (240) and eCBs may also be involved in rat leydig cell differentiation (241). In rat adipocytes, AEA increased differentiation through PPARγ and up-regulation of CB1 (138). The involvement of both AEA and 2-AG in the activity of human osteoblasts has also been recently described, in which AEA increased early cell differentiation, while 2-AG decreased late osteoblast-specific markers of differentiation (49).

For years, the “unpredictable” fate of eCB transduction cascade, mainly dependent on the stimulus and cell-type by modulation of a countless variety of signalling pathways motivated the studies on this field, showing that much more remains to be discovered. More recently, it has been highlighted that AEA-oxidative metabolites are also associated with programmed cell death, particularly in conditions where COX-2 is up-regulated. For instance, PME_2 and PMD_2, were associated with AEA-induced apoptosis in tumorigenic keratinocytes (242), in resistant colon cancer cells (151), and in JWF2 tumour cells that over-express COX-2 (243). Moreover, in tumorigenic keratinocytes, which overexpress COX-2, AEA-apoptosis was shown to be mediated by a novel J-prostamide, PMJ_2 resultant from COX-2 oxidative metabolism (167, 168), while in melanoma cells, it was reported that COX-2 and LOX metabolites mediate AEA-proapoptotic effects (244).

3. Human endometrium

A new life starts on the union between a sperm and an egg. The fertilized egg proliferates and undergoes several mitotic cell divisions eventually forming the blastocyst. The latter is comprised of two distinct cell types, the inner cell mass (ICM) that forms the embryo and the outer cell mass, called trophectoderm cells (TE), which contributes to the formation of trophoblasts and extra-embryonic tissues. Embryonic development depends on growth-factors secreted by the uterus, and upon contact with luminal epithelium, trophoblasts release chemotactic and invasive migration signals to the uterus. In turn, maternal endometrium undergoes an extensive remodelling process to allow implantation. Thus, the human endometrial stromal cells (HESCs) proliferate and differentiate into specialized decidual cells, a process named decidualization, to prepare a receptive endometrium. Notably, only for a short period of time, endometrial receptivity allows implantation, designated “window of implantation” (WOI) period (245, 246). In many species, decidualization of the stromal compartment is only observed upon blastocyst implantation. However, in humans, this process is initiated in the midsecretory phase of the menstrual cycle, independent of pregnancy. Besides humans, it occurs in a handful of species, including higher primates (apes, and old world monkeys), some bats and the elephant shrew. Impaired decidualization underpins a variety of reproductive disorders, ranging from implantation failure, recurrent miscarriage, pregnancy disorders and infertility.

The uterine *corpus* is composed of a modified mucosa known as the endometrium, a fibromuscular wall, the myometrium, and a serosal lining. The endometrium is composed by single-layered simple columnar epithelium, a thin basal layer (basalis), which bounds the myometrium, and a functional layer. In contrast to the basal layer, the latter is highly responsive to hormonal ovarian influence. The functional endometrium consists of a superficial layer with few glands and abundant stroma, and a deep layer that has many glands and relatively less stroma. In each menstrual cycle, the functional layer proliferates, secretes and sheds, whereas the basal layer provides the endometrial stem/progenitor cells that will regenerate the functional layer.

The major hormones that control uterine physiology are the ovarian steroids, progesterone and estrogens. The proliferative phase of the menstrual cycle is orchestrated by estradiol, and aims endometrial regeneration. It is characterised by re-epithelialization of endometrium, followed by the rapid renewal of the uterine glands and stromal compartment and repair of the vascular bed (247, 248). Ovulation marks the start of the progesterone driven luteal phase, which is characterized by cessation of

endometrial proliferation and the onset of stromal cell transformation (249). Differentiation of the stromal compartment represents a tipping point after which the fate of the endometrium becomes irreversibly dependent on progesterone signalling. In the absence of a conceptus, and on progesterone withdrawal, the functional layer undergoes cell death, infiltration of inflammatory cells and sloughs by menstruation (250, 251). In the case of pregnancy, persistently elevated progesterone levels ensure the extension of decidual reaction to the basal endometrial layer which is critical for trophoblast invasion and placenta formation (249).

3.1 Decidualization

The term ‘decidua’ is derived from the Latin ‘*decidere*’, meaning to die, to fall off or to detach. The decidualization process occurs in humans and few other species characterized by deep placentation that involves breaching of the luminal epithelium and invasion of maternal vascular network and of decidual tissue by fetal trophoblasts. This type of placentation, named hemochorial placentation, occurs in both primates and rodents, and underpins a close connection between maternal and fetal tissues (252). During early pregnancy, maternal uterine spiral arteries (SA) are remodeled to allow the transformation of an high resistance relatively hypoxic vascular bed into an increased oxygen tension, low resistance and high flow circuit (253). Uterine natural killer cells (uNK), the major maternal immune component of the decidua, accumulate around SAs before trophoblast invasion. Although not yet clarified, it was suggested that both uNK cells and fetal trophoblasts play important roles in vascular remodelling (254). In this regard, inadequate invasion by trophoblasts into uterine environment results in a deficient blood supply, which eventually leads to fetal prematurity, fetal growth restriction and pre-eclampsia (255). However, excessive invasion of uterine wall, which occurs in the absence or defective establishement of decidua, results in placenta creta (256).

From an evolutionary perspective the ´spontaneous´ decidualization likely evolved from the need of maternal tissue to protect from the onslaught of invasive trophoblasts or as a response to the ineficiency of human reproduction (257). Under maternal control, the differentiation process occurs cyclically each month, irrespective of pregnancy and no longer triggered by an implanting embryo. On the other hand, menstruation enables the activation of progenitor/stem cells and cyclic endometrial repair (258).

3.1.1 Decidualization: morphological and biochemical perspectives

Decidualization is characterized by transient local edema, influx of macrophages and uNK, angiogenesis and the extraordinary transformation of resident endometrial stromal fibroblasts into secretory decidual cells (249).

Morphologically, the non-differentiated endometrial stromal cells, have a fibroblast-like appearance with well-developed rough endoplasmic reticulum (ER) and Golgi apparatus, little and elongated cytoplasm (259, 260). When exposed to a decidual stimulus, these cells transform, both *in vivo* and *in vitro*, into larger cells, expanded cytoplasm accumulating glycogen and lipid droplets, a prominent nucleus and dilated ER and Golgi systems. In the late secretory phase, decidualizing stromal cells (DSCs) show pseudopodia-like extensions and possess phagocytic activity. In addition, intense changes are observed in the extracellular matrix composition (ECM) that becomes rich in type IV-collagen, laminin, decorin, fibronectin and heparan sulphate proteoglycans (261).

This morphological transition of stromal cells is accompanied by secretion of various factors, such as prolactin (PRL) and insulin growth factor-binding protein-1 (IGFBP-1) (262, 263), the most widely used decidual biomarkers to assess decidualization *in vitro*. The former has been suggested to stimulate trophoblast growth and invasion, promote angiogenesis, modulate uNK cell survival, prevent immune rejection and regulate water transport across the amnion (264-266). The role of IGFBP-1 is not fully clarified, though it has been postulated that it controls trophoblast invasion and regulates stromal cell growth and differentiation (247, 267, 268). Another factor released by decidualizing cells, is a member of the transforming growth factor-β superfamily, termed LEFTY, that appears to play a rate-limiting role in the extension of the decidual process (269, 270). Many other molecules have been described to act as paracrine/autocrine factors. These include key transcription factors such as forkhead box protein FOX01, the CCAAT/enhancer-binding protein (C/EBP1), the Hox genes (HoxA) (271-273), secretory products like, bone morphogenetic potein-2 (BMP2) and its downstream activator Wingless 4 (Wnt4) (274).

The microarray analysis has been used to elucidate the extension and magnitude of the molecular mechanisms underlying the decidualization process. On the basis of these studies, decidualization has been described as a process of sequential reprogramming of functionally related families of genes involved in cell cycle regulation, ECM organization, cell adhesion, cytoskeletal organization, angiogenesis, immune modulation of implantation, steroid hormone action and metabolism, stress response and apoptosis modulation (275, 276).

3.1.2 Differentiation pathways and deciduogenic cues

Initiation of the decidual process requires the increase of cAMP levels to sensitize estrogen-primed endometrial stromal cells to progesterone. Despite the importance of progesterone in maintaining the decidualized status, through transcriptional regulation of a variety of decidual proteins, it is not considered an initiation signal for this process (249, 277). Cyclic AMP is an ubiquitous second messenger generated upon activation of GPCR that leads to sustained activation of the PKA pathway (278). Upon binding of two cAMP molecules to each PKA regulatory subunit, the latter undergo a conformational change, which eventually results in the activation of several transcription factors (249). Several molecules that act through GPCR, particularly the G_s subunit, such as the luteinizing hormone/human chorionic gonadotrophin (LH/hCG), corticotrophin releasing hormone (CRH), relaxin (RLN), and PGE_2 are also involved in the onset of decidualization process by increasing cAMP levels (279-281). Once the decidual process is initiated, the DSCs, the epithelial cells, the local immune cells and the endometrial vasculature secrete a number of factors like cytokines, interleukins and growth factors, such as IL-11 and the leukemia inhibitory factor (LIF), members of the TGFβ superfamily including activins, inhibins, and follistatin and the transmembrane heparin-binding epidermal growth factor (HB-EGF) that are involved in the propagation of the differentiation signal (282-287).

In rodents, it is well established that cellular proliferation, differentiation, polyploidization and apoptosis occur in a spatiotemporal manner during the reproductive cycle and pregnancy. Decidual cell polyploidy is often characterized by the development of multinucleated giant cells, whose physiological significance is still unclear (288). It is speculated that polyploidy limits the life span of decidual cells to allow the growth of the implanting embryo (289). Hence, polyploid decidual cells are thought to be terminally differentiated cells that develop via an endoreplication cycle, involving cell mitoses without subsequent cytoplasmatic division, the cytokinesis (289, 290). Although several cell cycle regulators are involved in uterine decidualization, such as cyclins, cyclin-dependent kinases and their inhibitors, in rodents, cyclin D3 is the most studied. For instance, cyclin D3 has been identified as a downstream controller of several decidual regulators, such as the death-effector domain-containing protein (DEDD) and the homeobox A10 (Hoxa-10) (291-293). In humans, decidualization of HESCs was associated initially with cell cycle arrest at G_0/G_1 phase and $G_2/_M$ phase at later stages (294) and, although, the presence of bi-nucleated cells has been reported several years ago, their significance has never been addressed (295).

3.1.3 Functions of decidua

It is established that cyclic endometrial decidualization and menstrual shedding are an example of physiological preconditioning that prepares uterine tissue for the dramatic vascular remodelling, reactive oxygen species production and inflammation associated with deep placentation (296). The functions of decidua are summarized on Figure 11.

Decidualizing stromal cells transit from an initial acute pro-inflammatory response to an anti-inflammatory highly secretory response. The inflammatory phenotype renders an endometrium transiently receptive to implantation. While the mature secretory phenotype enables the endometrium the capacity to sensor embryo quality and to mount a tailored response to individual embryos in a manner that either supports further development or facilitates early rejection (297). Notably, it has been suggested that DSCs are able to recognize and eliminate developmental impaired embryos through a process similar to menstruation (298-300). This paradox of DSCs represents another hallmark of human decidualization, since they are programmed to resist a variety of environmental stressors in early pregnancy and to trigger tissue destruction in the absence of implantation or in response to a compromised embryo. On the other hand, although impaired decidualization does not prevent implantation, it predisposes to subsequent pregnancy failure by disabling natural embryo selection (298).

Once the luminal epithelium is breached, DSCs surround and encapsulate the invading conceptus. Hence, recent evidences demonstrate that decidualized endometrial cells produce a repertoire of matrix metalloproteinases (MMP) that endow an invasive capacity to these cells (301-303).

Furthermore, decidual tissue not only provides a favorable matrix for trophoblast expansion, allowing placental development and growth of the offspring, but also has the ability to regulate fetal invasion. DSCs also provide protection to inflammatory and oxidative insults, and modulate maternal immune response to fetal alloantigens (257). Early pregnancy is characterized by profound vascular remodelling and fluctuations in oxygen tension at the maternal-fetal interface. Initially, the fetoplacental interface develops in a low-oxygen environment, protecting the embryo against reactive oxygen species (ROS). However, decidual compartment underpins resistance to oxidative-stress induced apoptosis, including increased free radical scavenging, selective silencing stress MAPK pathways and inhibition of FOXO3a, a pro-apoptotic transcription factor that mediates oxidative cell death in undifferentiated stromal cells (304).

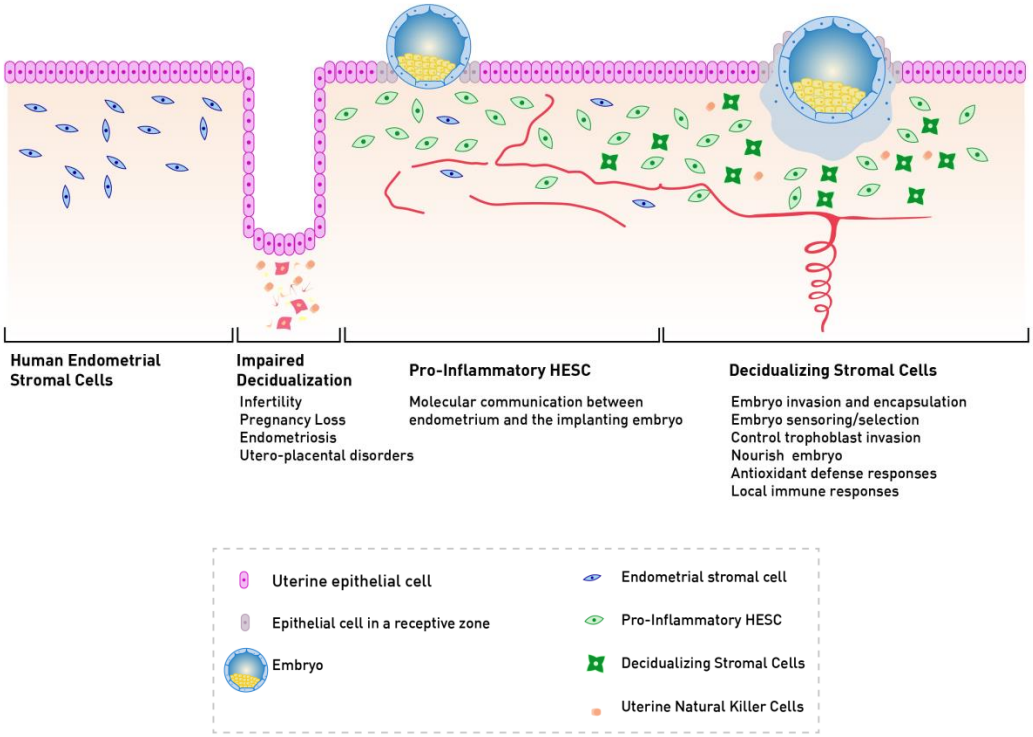

Figure 11. Decidual transformation of human endometrial stromal cells (HESCs). The decidual process transits from an acute inflammatory initiation phenotype (pro-inflammatory HESC) to an anti-inflammatory, highly secretory phenotype (decidualizing cells). This transformation underpins the acquisition of specialized functions.

3.1.4 Decidualization in rats

In rodents, decidualization is triggered by a physic stimulus, blastocyst implantation, though it can also be artificially induced in pseudopregnant and ovariectomized rats, giving rise to deciduoma. This suggests that the decidual response is an intrinsic program of uterine stromal cells, not influenced by embryo (305). Intra-luminal infusions of oil or scratching with a needle are widely used as a decidual stimulus (306). Figure 12 depicts a schematic representation and a timeline of day-specific morphological changes of rat uterine tissues along pregnancy.

At the site of blastocyst implantation, endometrial fibroblast-like stromal cells proliferate and differentiate into decidual cells, which consequently dye by programmed cell death (305, 307, 308). The pattern of the decidualization is not simultaneous, but rather a dynamic temporal and spatial sequence of events, occurring in different regions at different times.

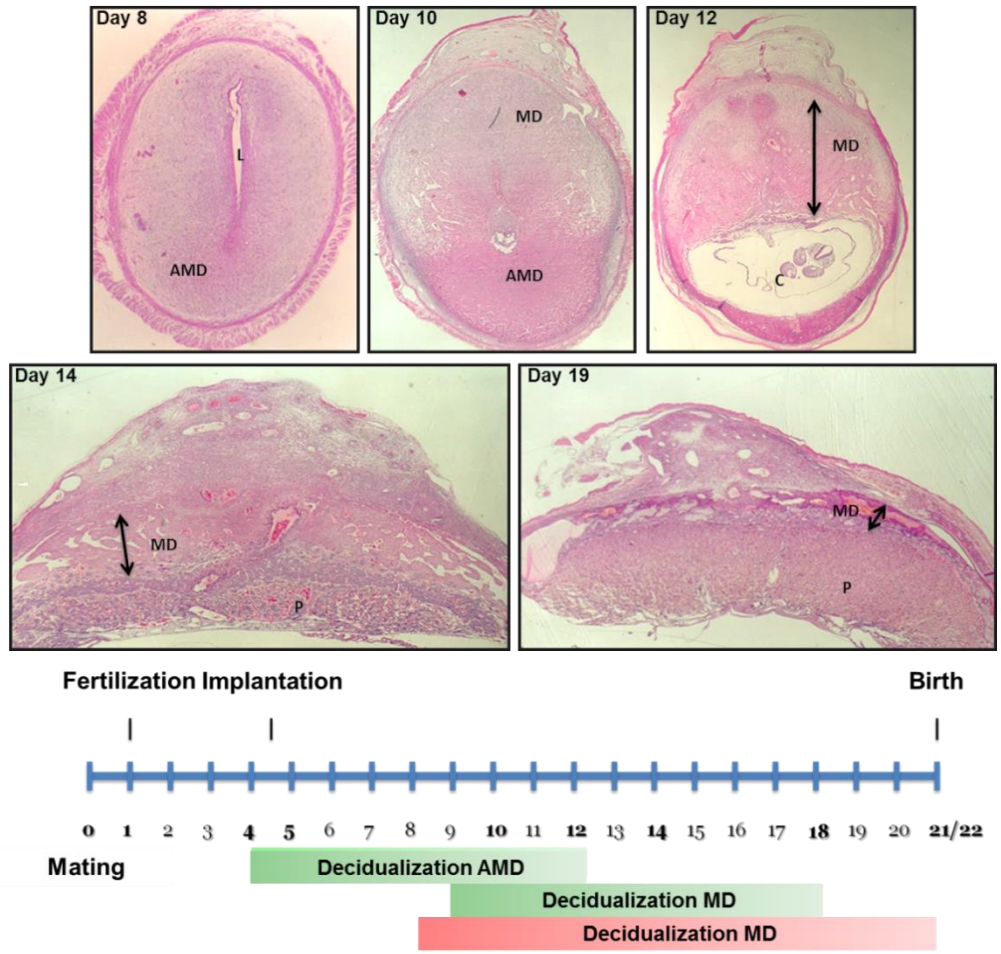

Figure 12. Schematic representation of rat uterine tissues morphological alterations and timeline of day-specific morphological characteristics along pregnancy. D1 of pregnancy is confirmed by the presence of spermatozoa in rat vagina smear. Implantation occurs between d4/5 at the anti-mesometrial side of the uterus and is followed by a fast growth of the feto-placental unit. Decidualization process begins antimesometrially after blastocyst attachment. Degeneration of this tissue starts at d10 and by d12 it has completely regressed. Alongside, mesometrial pole begins to differentiate on d9. After d12, when it reaches its maximum development, mesometrial decidua progressively regresses to allow placental growth. Meanwhile invading trophoblast cells breach the uterine basement membrane to infiltrate the decidua, which will lead to formation of the placenta. AMD: antimesometrial decidua; L: Lumen; MD: mesometrial decidua; P: placenta.

In the rat, decidualization begins in the vicinity of the implanting blastocyst, on the antimesometrial pole, giving rise to the antimesometrial decidua (AMD). Parallel, epithelial cells at the site of blastocyst apposition progressively undergo apoptosis. Between days 5-6 of pregnancy, stromal cells surrounding the implanting embryo cease to proliferate and undergo differentiation into polyploid decidual cells, forming an avascular and dense zone termed the primary decidual zone (309). These cells are large, round or polygonal with basophilic cytoplasm, two or more nucleus with prominent nucleoli. By day 8, the decidual reaction reaches the basal zone adjacent to the circular muscle coat, achieving its maximum development at day 10. Subsequently, it progressively regresses by

apoptosis and secondary necrosis, forming the decidua capsularis (310). Parallel, endothelial cells proliferate rapidly in the lateral wing of the uterus, resulting in the formation of large venous sinusoids, in a region called glycogenic wing area, which appear to intervene in the transfer of nutrients (308).

Thereafter, on day 9, differentiation extends to the opposite side of the uterus, named the mesometrial side of the endometrium, comprising the mesometrial decidua (MD) (311, 312). Mesometrial decidual cells are smaller than those of the antimesometrial decidua, irregular in shape and contain a single nucleus. The MD reaches its maximum development on day 12 of gestation, and upon day 14 it begins to regress by programmed cell death, allowing fetoplacental development, and giving rise to the decidua basalis. The latter forms the maternal component of placenta and persists till the end of pregnancy. By day 14 the hemochorial placenta is fully established.

Simultaneously to the development of mesometrial decidua there is the appearance of uNK population (313). Like in humans, uNK cells are phenotypically and functionally different from circulating NK cells, they express integrins, which allow their migration towards the decidualizing endometrium (314, 315). After maturation, they acquire a granular phenotype containing perforin, granulysin and granzyme B, contributing to maternal vascular remodelling and angiogenesis (315, 316). By day 12 these cells also appear in the mesometrial triangle, a region between the circular and longitudinal muscle coat, through which the blood vessels gain access to the uterine endometrium (313). After, uNK cells degranulate and die by apoptosis. The mesometrial triangle region enlarges during the second half of pregnancy to give rise to the metrial gland.

Degeneration of both antimesometrial and mesometrial decidua is a remarkable feature of rat placentation. AMD has all the characteristics of an endocrine organ, secreting a variety of hormones and growth factors, such as decidual prolactin-like hormones, follistatin, activin and transforming growth factors (317). MD regression is intimately related with trophoblast invasion and placental development. The main secretory product of rat mesometrial decidua is a glycoprotein termed 'decidualization-associated protein', which shares identical properties with the acute phase protein α2-macroglobulin, a general protease that has the ability to bind to all types of proteases and numerous growth factors, controlling in that way their activity (318). Although it is not present in human endometrium, in rodents this protein is important for spiral arterial modification and prevention of excessive trophoblastic invasion (319).

4. Endocannabinoids and reproduction

The hazards of *C. sativa* consumption in reproduction are known for decades. THC inhibits ovulation, suppresses the production of sex hormones, crosses placenta, accumulates in maternal milk, and impairs pregnancy outcome (320). Prenatal use has been associated with infertility, placental complications, low birth weight, preterm labor and fetal growth restriction. Whereas long-term effects of prenatal marijuana use on exposed offspring include poorer executive functioning skills and attention, increased conduct and behaviour problems, and poorer school achievement (321). In men, studies have reported the role that marijuana plays in disrupting spermatogenesis, and sperm function such as motility, capacitation, and the acrosome reaction (322). Nevertheles, the mechanisms underlying these adverse effects remains unclear.

In the past few years, the ECS has emerged as an essential player in male and female reproduction. eCBs together with their metabolic enzymes and molecular targets have been identified in reproductive cells, organs, and fluids of invertebrates, vertebrates and mammals, highlighting a key role in reproduction processes along the evolutionary axis. In fact, CB1 receptor is expressed in the embryo, in much higher levels than those in the brain, and, among the reproductive tissues, the endometrium is the major source of AEA (323, 324).

4.1 The Endocannabinoid System and early pregnancy

It is widely known that endocannabinoids are involved in all stages of female reproduction, from oocyte development to parturition (325). Figure 13 resumes eCBs effects on pregnancy events.

Besides cannabinoid receptors, preimplantation mouse embryo, as well as the oviduct and the uterus, possess the full enzymatic machinery to synthesize and degrade AEA. In mice, CB1 mRNA is detected from the 4-cell embryo, while CB2 mRNA is present from the zygote to blastocyst stage (323, 326). While CB1 receptor is the functional receptor responsible for normal embryo growth and development, CB2 controls stem cell populations (327, 328). In fact, it has been demonstrated that CB1 activation inhibits embryo development *in vitro* (326, 329). At the same time, while NAPE-PLD is present from the early fertilized egg to the blastocyst stage, FAAH expression was detected later at the 2-cell embryo, suggesting a protective role against AEA cytotoxicity (330).

During early pregnancy, CB1 plays another critical role in the transport of embryos from the oviduct to the uterus. In CB1 $^{-/-}$ mouse, embryos are retained in the oviduct and fail implantation, due to an impairment of oviduct smooth muscle contraction and relaxation required for embryo transport (331, 332). Accordingly, the oviductal transport of the fertilized egg and its development to the competent stage are controlled by a gradient of AEA concentration, which increases from the ampulla to isthmus. The appropriate local tone of AEA is controlled in the uterus by the differential expression of NAPE-PLD and FAAH and is conducive to a successful implantation (332). In this respect, FAAH $^{-/-}$ mice exhibit retarded embryo development and also altered oviductal transport (333, 334).

Synchronized embryo development and uterine differentiation are crucial for implantation, and it is well established that a deregulation of the eCB signalling disrupts both processes (331). Down-regulated levels of AEA detected at implantation sites are associated with uterine receptivity, while high AEA levels are found in the non-receptive uterus, refractory to implantation, and at inter-implantation sites (324, 326). In addition, this eCB also plays an important role in rat decidual remodelling and stromal cell differentiation (65, 213, 239). On the other hand, eCB signalling is also implicated in blastocyst development. In this respect, CB1 expression is lower in the activated blastocyst than that in dormant blastocysts (57, 335). Furthermore, through CB1 binding, low AEA levels stimulate blastocyst differentiation and trophoblast outgrowth, while high levels inhibit embryo development and zona-hatching of blastocysts. These dual effects of AEA depend on the activation of different signal transduction pathways. While low levels of AEA activate ERK signalling pathway, higher concentrations inhibit Ca^{2+} influx (324, 328).

Altogether these observations support a role for the endocannabinoid signalling in embryo synchronization with the uterine receptivity. Although still controversial, it has been reported that mouse blastocysts secrete a molecule, not yet identified, that regulates uterine activation of FAAH activity and inibits NAPE-PLD (336, 337). Hence, FAAH emerges as the main metabolic gatekeeper controlling AEA tone and CB1-activated pathways to ensure early pregnancy events.

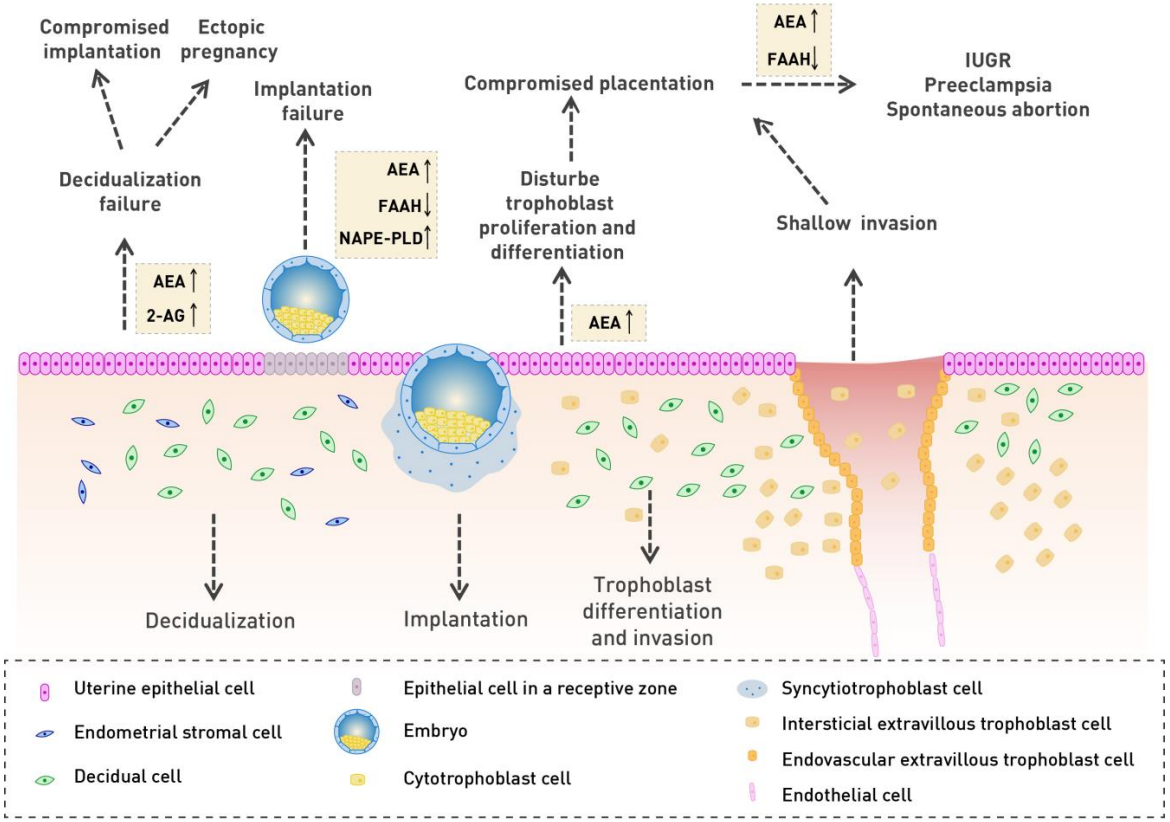

Figure 13. Schematic representation of the fetomaternal interface and potential adverse effects arising from deregulated endocannabinoid signalling based on rodents and human studies. Endometrial stromal cells differentiate into decidual cells, preparing uterine tissues for pregnancy, whereas the invading trophoblast cells critically regulate placental growth and function. All the physiological and molecular processes occurring during those periods are complex but highly organized. Endocannabinoids have reported to be involved in decidualization, implantation, trophoblast differentiation and invasion. Aberrant endocannabinoid signalling (shown in yellow boxes) is reflected in compromised reprogramming of the endometrial stromal cells, implantation and placentation manifesting in ectopic pregnancy, intrauterine growth restriction, preeclampsia, miscarriage, and spontaneous abortion. Adapted from (338).

In humans, cannabinoid receptors are active at the ovaries in cortex and medulla. In contrast, FAAH and NAPE-PLD are only expressed in secondary and tertiary follicles, corpus luteum and corpus albicans, but not in oocytes (339). Interestingly, in the follicular fluid AEA levels correlated with follicle size, being lower in follicles from which oocytes are not retrieved, thus indicating that AEA may be involved in follicular maturation and development (340).

The expression of the components of the ECS has also been reported in the human endometrium. CB1 expression is higher in endometrial glands than in the stromal compartment, and it was found that CB1 mRNA and protein levels are increased in the secretory phase likely due to progesterone modulation (341, 342). CB2 expression is minimal at the beginning of the cycle and reaches a peak during the late proliferative phase (341). At the secretory phase, besides increased FAAH expression in the decidual compartment, it has been recently described that COX-2 expression is also high,

suggesting that together, these enzymes may promote an appropriate eCB tone for decidualization and a successful implantation (343). Regarding NAPE-PLD expression during the menstrual cycle there is still some controversial information (343, 344). Throughout the menstrual cycle, plasmatic AEA levels fluctuate. High AEA levels are found in the follicular phase, peaking at ovulation and, subsequently decreasing towards the luteal phase, which coincides with the “window of implantation” (333, 339). Altogether these observations reinforce that high levels of AEA in ovulation and lower levels in implantation period are required for a successful pregnancy.

During the menstrual cycle, a link between AEA and FSH during the follicular phase and between AEA, LH and estradiol during ovulation was observed (345). In this way, it has been suggested that eCBs may modulate reproductive function both locally and at the hypothalamic–pituitary–gonadal axis (346). In addition, it has been demonstrated that FAAH regulation in the peripheral lymphocytes, both at activity and expression levels, is a key point during menstrual cycle. Under the control of progesterone, high FAAH expression/activity and concomitant decreased AEA levels characterize the “implantation window” (347).

Recently, it has been reported that eCBs also control endometrial plasticity by regulating stromal cell motility and migration via CB1-dependent activation of Akt and ERK1/2 pathways (348). Also, methanandamide enhances HESC proliferation and induces apoptosis in longer exposures (346). However, still little is known about the role of eCBs in proliferation and differentiation of HESCs. Nevertheless, it has been reported that the CB1 agonist, WIN 55212-2, exerts an anti-proliferative action on stromal endometriotic cells linked to a reduction of ROS production and Akt pathway inactivation (349). On the other hand, it was found that CB1 expression, both at mRNA and protein levels, was lower in endometriosis, suggesting that reduced eCB signalling correlates with enhanced proliferative capacity of endometriotic lesions (342). Besides, WIN 55212-2 through CB1 binding, inhibited human decidualization and stimulated apoptosis by a cAMP-dependent mechanism (350).

It has been described that the interplay of sex steroids, cytokines and eCBs result essentially from the modulation of FAAH, the major controlling enzyme of AEA, by oestrogen, progesterone, leptin and cytokines, indicating a role for eCBs-hormone-cytokines in the regulation of human fertility (330). Disruptions in the endocannabinoid system, especially a shift in the balance between AEA levels and FAAH activity have been implicated in human implantation disorders and infertility (345, 351). Lower CB1 mRNA expression was found in fallopian tubes and decidua of women with ectopic pregnancies compared to normal pregnancy (352). In addition, aberrant AEA levels and reduced CB1 and FAAH expression in fallopian tubes, as well as decreased FAAH activity in peripheral

blood cells were reported in women with ectopic pregnancy (353, 354). In women undergoing *in vitro* fertilization (IVF)/intracytoplasmic sperm injection (IVF/ICSI)-embryo transfer, it was verified that AEA plasma levels decreased from the day of oocyte retrieval to that of the embryo transfer in pregnant women. Thus, it was suggested that low serum AEA levels at the time of implantation was a sign of a successful pregnancy (345, 355, 356). Furthermore, low FAAH and high AEA levels in blood are associated with spontaneous miscarriage, as reported in two studies where low FAAH activity was found in lymphocytes of women who spontaneously aborted (357, 358). Trabucco *et al.* (2009) investigated placental tissue from women after spontaneous miscarriages and identified low FAAH expression and increased CB1 expression (359). As occurs in rodents, these studies reinforce a biphasic role for AEA, under FAAH control, in regulating both blastocyst development and transport with uterine receptivity and implantation.

During the first and second trimesters of pregnancy, plasma AEA levels are comparable to those of luteal phase, consistent with successful pregnancy maintenance. Then it significantly rises at term and further during labor (360, 361). Therefore, AEA plays a role in folliculogenesis, follicle maturation, oocyte maturation, ovulation, implantation, decidualization and early pregnancy (Table 1).

Table 1. Overview of the most important biological action of AEA in the female reproductive organs.

Oviduct	• Oviduct contraction • Embryo transport
Ovary	• Oocyte maturarion • Folliculogenesis
Endometrium	• Embryo implantation • Endometrial cell motility and proliferation • Decidualization

4.2 Decidualization: the affair between endocannabinoid and prostaglandin network

Decidualization is a remarkable feature of human reproduction, involving the cross-talk of a complex network of hormones, cytokines and interleukins, transcriptional and growth-factors. In rat uteurs, the interaction between lysophosphatidic acid (LPA), PGs, and ECS during the window of implantation has been reported (362), although in humans it is not yet elucidated.

A growing body of evidences, supported by studies using the rat as a model, indicates that anandamide has an important role in the determination of endometrial cells' fate. Elevated AEA levels induced apoptosis in rat decidual cells, via CB1-dependent p38 activation (65, 213). In addition, CB1 expression was highest during the maximum development of the mesometrial decidua (363). Regarding metabolic enzymes, it has been observed that COX-2 and FAAH expression decrease after day 14 of pregnancy, which coincides with decidual regression, reinforcing the role of AEA on decidual cell turnover. Interestingly, the plasmatic AEA and decidual tissues levels do not correlate, suggesting an *in situ* regulation (364, 365). Furthermore, it has been recently shown that AEA limits uterine stromal cell differentiation and critically impairs decidualization (239).

Besides eCBs, other pivotal lipid mediators of decidual process are currently (re)born in the literature. Not only they mediate a tailored role in the decidualization process, but foremost a cross-talk between these molecules is probable. PGs are believed to play a crucial role in the regulation of endometrial vascular permeability, angiogenesis and decidualization, trophoblast invasion and extracellular matrix remodelling during implantation (366). In fact, increased vascular permeability is an essential prerequisite for decidualization in rodents and is attributed to PGE_2 (367, 368). Cytosolic phospholipase A2a (cPLA2a) is a major provider of AA for COX-2. Interestingly, cPLA2α-null mice failed in implantation (369). In rats, COX-2 is mostly restricted to implantation sites, and COX-$2^{-/-}$ mice are infertile, with defective implantation and decidualization (370, 371). In addition, COX-2 expression in epithelium and stromal compartment is increased after blastocyst implantation and PGE_2 levels are also higher during the period of decidual development (371). Blockade of PG synthesis before or during implantation causes a complete inhibition, a delay in implantation or a reduction in the number of implantation sites and impaired decidual response. However, exogenous administration of PGs can partially restore these processes (372, 373). COX-2-derived PGI_2 appears to play an important role in protecting preimplantation embryo from apoptosis (374).

In humans, the regulatory role of PGs in early pregnancy events is emerging. In fact, these molecules were recently perceived as potential biomarkers of endometrial receptivity and pregnancy failure, since PGE_2 and PGF_2 levels are significantly increased during the receptive phase (375). Moreover, a deregulation of the PG signalling has been correlated to poor endometrial receptivity, infertility and uterine pathologies (376, 377). On the other hand, PGE_2 may act through prostaglandin receptors, increasing intracellular levels of cAMP and stimulating the activity of alkaline phosphatase, both factors critical for decidualization (281, 378). These observations highlight the role of COX-2 derived prostaglandins in early pregnancy events. In fact, arachidonic acid available from AEA hydrolysis by FAAH may give rise to PGs. Nevertheless, COX-2 may also intervene

during decidualization process by producing AEA-oxidative metabolites, the prostamides. On one hand, in the rat uterus, it has been suggested that eCBs mediate the stimulatory effect of LPA on COX-2 derived PGE_2 production (362). In contrast, it has been reported that AEA exerts opposite effects on PGE_2 and $PF_{2\alpha}$ in mice uterine explants, by inhibiting production of the former and increasing the levels of the latter (379). In addition, PGs-mediate the AEA-inhibitory action on nitric oxide synthase activity in the receptive rat uterus (380). Fonseca *et al.* (2015), recently unveiled that the inhibitory role of AEA in uterine stromal cell differentiation may involve COX-2 down-regulation (239). While in humans, AEA modulates production of PG in fetal tissue, by a CB1 dependent manner (381).

The fact that, in rats, pregnancy uterine tissue levels do not correlate with plasma eCBs levels, suggests that during pregnancy, maternal tissue levels of AEA are regulated by *in situ* production and degradation to create an appropriate eCB gradient conducive to a successful pregnancy (364). In this respect, prostamides may be novel lipid mediators of some endocrine and/or paracrine effects of eCBs. Nevertheless, both in humans and rodents, high FAAH expression/activity and low AEA levels correlates with the rise in PGs levels and COX-2 expression during the secretory phase of menstrual cycle and uterine receptivity (338, 343). It may be that, FAAH and COX-2 play together a crucial role in protecting the embryo from deleterious AEA effects at implantation, but also in regulating decidual remodelling and endometrial receptivity (Figure 14). The notable expression of COX-2 contiguous to embryo, in rats, contrasting with FAAH expression, also highlights the importance of COX-2 (364). Therefore, compelling evidences suggest the intimate coordination between eCB and PGs during early pregnancy.

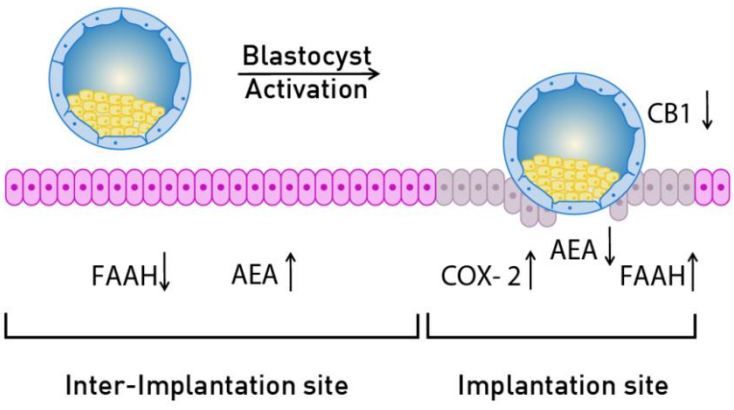

Figure 14. Endocannabinoid signalling during blastocyst implantation. Regulated levels of AEA in the receptive uterus and CB1 in activated blastocysts at the time of implantation are beneficial for implantation, whereas higher levels are detrimental to this process. High FAAH and COX-2 expression is found in the implantation sites. Because COX-2 is expressed in the uterus at the site of implantation and AEA may serve as substrate for either COX-2 or FAAH, the proposal suggests that uterine eCBs are tightly regulated by the coordinated activity of FAAH and COX-2 in the uterus during early pregnancy. AEA: anandamide; Cyclooxygenase-2: COX-2; FAAH: fatty acid amide hydrolase. Adapted from Dey et al. (2004) (306).

Aims of the study

Endometrial plasticity is a remarkable feature in the biology of reproduction. Human endometrial stromal cells undergo a balance remodelling involving proliferation, differentiation and apoptosis, and an impairment of these events may result in pregnancy loss, miscarriage and infertility.

Over the last decade, endocannabinoids emerged as key players in the control of cell fate in several physiological processes. In the reproductive system, a strictly regulated anandamide tone enables embryo transport and implantation, endometrial receptivity, fetoplacental development and labor. We have previously described that, in rats, AEA impairs the progression of stromal cell differentiation and induces apoptosis of decidual cells. On the other hand, in rats, COX-2 is recognized as a major enzyme involved in implantation and decidualization and COX-2-derived metabolites may mediate some eCBs-induced effects. Therefore, the latter may be active players under conditions of enhanced eCB synthesis, as in the case of *Cannabis sativa* derivates consumption, or in pathological conditions-related to ECS deregulation. Since the understanding of the oxidative metabolism of endocannabinoids might also delineate a new world of signalling pathways, one aim of this work is to unravel the role of COX-2 metabolism and derived AEA-metabolites in rat decidual cell cultures.

In humans, poorly defined cellular and molecular mechanisms underpin the role of eCBs in the control of endometrial stromal cell remodelling. In fact, dissecting the regulators and the molecular basis underlying human endometrial cell turnover may shed light on the pathophysiology of some pregnancy disorders and/or infertility. Thus, another aim of this project is to characterize the ECS in human endometrial stromal-derived cells and to study the impact of AEA in HESCs proliferation, apoptosis and differentiation. In addition, we hypothesize that COX-2 may be the ‘lock-key’ to switch the balance between eCBs and PGs, favoring, embryo implantation and endometrial receptivity. Therefore, to decipher the latent cross-talk among these two major lipid mediators of reproductive biology, the impact of a deregulation of eCB signalling on COX-2 expression and PGs release during decidualization will be studied.

CHAPTER II

Experimental section

Manuscript I:

Lipidomic approach towards deciphering anandamide effects in rat decidual cell.

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Lipidomic Approach Towards Deciphering Anandamide Effects in Rat Decidual Cell

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Altered phospholipid (PL) metabolism has been associated with pregnancy disorders. Moreover, lipid molecules such as endocannabinoids (eCBs) and prostaglandins (PGs) are important mediators of reproductive events. In humans, abnormal decidualization has been linked with unexplained infertility, miscarriage and endometrial pathologies. Anandamide (AEA), the major eCB, induces apoptosis in rat decidual cells. In this study, the PL profile of rat decidual cells was characterized by a Mass spectrometry (MS) based lipidomic approach. Furthermore, we analyzed a possible correlation between changes in PL of rat decidual cells' membrane and AEA-induced apoptosis. We found an increase in phosphatidylserine and a reduction of cardiolipin and phophatidylinositol relative contents. In addition, we observed an increase in the content of alkyl(alkenyl) acylPL, plasmalogens, and of long chain fatty acids especially with high degrees of unsaturation, as well as an increase in lipid hydroperoxides in treated cells. These findings provide novel insights on deregulation of lipid metabolism by anandamide, which may display further implications in decidualization process.

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Reproduction in humans is marred by early pregnancy failure. Approximately 15% of clinically recognized pregnancies fail (Rai and Regan, 2006). Implantation failure is common following natural conceptions and is an important clinical hurdle to overcome following assisted reproductive attempts. Essential changes have to occur in endometrium to support implantation and establishment of pregnancy. Human endometrium is receptive to embryo for a short period of time, referred to as the "window of implantation." Hence, uterine endometrial stromal cells undergo a characteristic reaction to form transient decidual cells, termed decidualization. (Gellersen and Brosens, 2014) Abnormal decidualization predisposes to pregnancy complications, including unexplained infertility, miscarriage, fetal growth restriction and endometrial pathologies such as endometriosis (Laird et al., 2006).

Lipid molecules such as endocannabinoids (eCBs), lysophosphatidic acid (LPA) and prostaglandins (PGs) are relevant mediators of early pregnancy events (Sordelli et al., 2012). In addition to the well-known structural features and regulatory functions, lipids are also signaling molecules involved in many cellular processes such as cell growth, differentiation and apoptosis. Furthermore, deregulation of lipid metabolism is currently emerging among adverse pregnancy outcomes (Vilella et al., 2013). In fact, increasing evidence suggests that anandamide (AEA), the major endocannabinoid (eCB), has regulatory functions during implantation, decidualization and fetoplacental development (Fonseca et al., 2013a; Meccariello et al., 2014). AEA, among other endogenous cannabinoids, acts mainly through G-protein coupled receptors or GPCRs, termed cannabinoid receptors (CB), which along with the metabolic enzymes form the endocannabinoid system. Currently, it is well established that to achieve a receptive phase, low AEA levels in uterine environment are maintained by *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), the enzyme responsible for its synthesis, and by fatty acid amide hydrolase (FAAH), which control its degradation to arachidonic acid (AA) and ethanolamine (EA) (Wang et al., 2007; El-Talatini et al., 2009). Implications of the harmful effects caused by high AEA levels involve lower FAAH protein levels and activity in women who miscarry and those who fail in-vitro-fertilization (IVF) treatment (Maccarrone et al., 2000, 2002; Habayeb et al., 2008; El-Talatini et al., 2009). We have previously described that ECS operates in rat decidual cells and demonstrated the upcoming role of AEA in uterine remodeling process (Fonseca et al., 2009, 2013b). Particularly, we showed that AEA through CB1 receptor induces apoptosis in rat decidual cells (Fonseca et al., 2009). Furthermore,

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aberrant eCB signaling has been associated with decidua-related pregnancy disorders (Horne et al., 2008).

Recently, the development of lipidomic tools have enable the identification of potential biomarkers of endometrial receptivity towards the understanding of clinical disorders and forecasting pregnancy outcome (Vilella et al., 2013). Proteomic analysis of plasma from preeclamptic pregnancies showed the differential expression of several proteins mainly related to lipid metabolism and extracellular matrix remodeling (Blumenstein et al., 2009; Liu et al., 2011). On the other hand, lipidomic studies allowed lipid characterization of human endometrial fluid, and showed that PGs were significantly increased at the window of implantation/receptive phase (Vilella et al., 2013). Analysis of the phospholipid molecular species in human placenta showed a plasmenylphosphatidylethanolamine deficiency, as well as an increase of free fatty acids (Jain et al., 2004) and phospholipid levels in pre-eclamptic pregnancies (Huang et al., 2013). Also, increased contents of phospholipids, lipid peroxides and cholesterol was found in the decidua basalis of women with pre-eclampsia (Staff et al., 1999).

The molecular signaling network that coordinates successful strategies for decidualization is not well understood. Thus, in an attempt to better appreciate the cellular membrane dynamics underlying the decidual remodeling and the effect triggered by AEA-induced apoptosis we analyzed the phospholipid (PL) profile of rat decidual cells, in the absence and presence of AEA, using a MS based lipidomic approach. The alterations on phospholipid composition and levels may manifest a source of AEA-induced effects during uterine remodeling.

Materials and Methods

Animals and cell cultures

All animal experiments were conducted with Direção-Geral de Alimentação e Veterinária (DGAV) approval and conformed to the European legislation on the use of laboratory animals. Female Wistar rats weighing 200–250 g (Charles River Laboratories, Barcelona, Spain) were mated and the day on which spermatozoa were found in the morning vaginal smear was designated day 1 of pregnancy. Primary cultures of decidual cells were prepared as previously described (Fonseca et al., 2009). Briefly, mesometrial decidua was dissected from antimesometrial decidua and digested with dispase II (2.4 U/ml) (Roche Diagnostics GmbH, Mannhein, Germany), colagenase (50 U/ml) (Sigma Chemical Co, St. Louis, MO) and Dnase I (200 U/ml) (Roche Diagnostics GmbH, Mannhein, Germany) in HBSS (Gibco/Invitrogen Corporation, Carlsbad, CA) for 1 h at 37 °C. Non-digested tissue was removed by filtration and decidual cells were collected by centrifugation at 200 g for 10 min. Cells were cultured in 21 mm culture dishes at 1.2×10^6/ml in Dulbecco's minimum essential medium (DMEM) (Gibco/Invitrogen Corporation, Carlsbad, CA) supplemented with antibiotic-antimicotic solution (200 U/ml penicillin G, 0.5 μg/ml amphotericine B and 200 μg/ml streptomycin) (Gibco/Invitrogen Corporation, Carlsbad, CA) and 10% FBS (Gibco/Invitrogen Corporation, Carlsbad, CA) under a 95% air-5% CO_2 humidified atmosphere for 4 h. After adhesion, medium was removed and cells were cultured for 24 h in DMEM without FBS in the presence or absence of anandamide (AEA), 10 μM. Purity of cultures was evaluated by cellular morphology and immunocytochemistry for detection of α2-macroglobulin, the major protein synthesized by the mesometrial decidual cells.

Lipid extraction

Cells were detached by scraping and cell suspension centrifuged 10 min at 720 g. The cell pellet was washed with PBS, and centrifuged 4 min at 720 g. The final pellet was resuspended in distilled water (dH2O). Total lipids were extracted by the method described by Bligh and Dyer (Bligh and Dyer, 1959). Briefly, 3.75 ml chloroform/methanol 1:2 (v/v) was added to the sample (1 ml), vortexed, and incubated on ice for 30 min. An additional volume of 1.25 ml chloroform and of 1.25 ml dH2O were added, and following vigorous vortex, samples were centrifuged at 180 g for 5 min at room temperature to obtain a two-phase system: aqueous top phase and organic bottom phase from which lipids were obtained.

Phospholipid quantification

Total amount of PL as well as each PL class separated by TLC were quantified with a phosphorus assay, performed according to Bartlett and Lewis (Bartlett, 1959). To quantify the total PL extract, 5% of sample was used, and dried with a nitrogen flow. Following TLC separation, the spots corresponding to the different PL classes were scraped off directly to quantification tubes. Perchloric acid (70%) was added to samples, which were then incubated 1 h at 180 °C. To all samples dH_2O (3.3 ml), 1% ammonium molybdate (0.5 ml) and 4% ascorbic acid (0.5 ml) were added, followed by incubation for 10 min at 100 °C in a water bath. Standards from 0.1 to 2 μg of phosphate underwent the same treatment and absorbance was measured at 800 nm. The relative abundance (%) of each PL class was calculated by the ratio between the amount of PL in each spot and the total amount of PL in the sample. Prior to spectrophotometric quantification, the Thin Layer Chromatography (TLC) separated PL classes were centrifuged 5 min at 2900 g to disperse PLs from silica. Three experiments were analyzed in triplicate.

Separation of phospholipid classes by thin layer chromatography (TLC)

PL classes from the total lipid extract were separated by TLC using silica gel plates with concentrating zone 2.5×20 cm (Merck) as described previously (Doria et al., 2012, 2013). First, plates were treated with 2.3% boric acid in ethanol. Then, 20 μl of PL extract (20–30 μg of PL) dissolved in chloroform were seeded in the TLC plate. Spots were developed in solvent mixture chloroform/ethanol/water/triethylamine (35:30:7:35, v/v/v/v). Lipid spots on TLC plates were observed by exposure to primuline (50 μg/100 ml acetone:water, 80:20, v/v), and visualized with a UV lamp ($\lambda = 254$ nm). Identification of the different classes of PLs was carried out by comparison with the use of PL standards that run side by side on the TLC plate and confirmed by mass spectrometry analysis of each spot.

Fatty acid analysis by gas chromatography with flame ionization detection (GC-FID)

The total fatty acyl substituents were measured by GC after transesterification of the total lipid extracts (approximately 30 μg of total PL). The fatty acids methyl esters were prepared using a methanolic solution of potassium hydroxide (2.0 M) according to the previously described method (Aued-Pimentel et al., 2004). Hexane solution containing methylated fatty acids were submitted to GC analysis. The GC injection port was programmed at 523.15 K and the detector at 543.15 K. The oven temperature was programmed as follows: initially stayed 3 min at 323.15 K, raised to 453.15 K (25 K min-1), held isothermal for 6 min, with a subsequent increase to 533.15 K (40 K min-1) and maintained there for 3 min, performing 19 min totally. The carrier gas was hydrogen flowing at 1.7 ml min^{-1}. The gas chromatograph (Clarus 400, PerkinElmer, Inc., MA) was equipped with DB-1 column with 30 m length, 0.25 mm internal diameter and 0.15 μm film thickness (J&W Scientific, Agilent Technologies, Folsom, CA) and a flame ionization detector. The relative concentrations of fatty acids were calculated by the percent area method with proper normalization

considering the sum of all areas of the identified fatty acids (Aued-Pimentel et al., 2004).

Separation of phospholipid classes by high performance liquid chromatography mass spectrometry (HPLC-MS)

PL classes were separated and analysed by HILIC-LC–MS, performed on an HPLC system (Waters Alliance 2690) coupled to an electrospray (ESI) linear ion trap mass spectrometer (ThermoFinnigan, San Jose, CA). PL classes were analyzed by LC-ESI-MS in positive mode (PC, SM and PE classes) with formation of $[MH]^+$ and in negative mode (PI, PA and CL classes) with formation of $[M\text{-}H]^-$ (ThermoFinnigan, San Jose, CA) (Doria et al., 2012, 2013). HILIC conditions were: the mobile phase A consisted of 10% water and 55% acetonitrile with 35% (v/v) methanol. The mobile phase B consisted of acetonitrile 60%, methanol 40% with 10mM ammonium acetate. Total lipid extract were diluted in the mobile phase B and 15 μl of reaction mixture was introduced into an Ascentis Si HPLC Pore column (15 cm × 1.0 mm, 3 μm) (Sigma–Aldrich). The solvent gradient was programmed as follows: gradient started with 0% of A and linear increased to 100% of A during 20 min, and held isocratically for 35 min, returning to the initial conditions in 5 min. The flow rate through the column was 16 μL/min obtained using a pre-column split (Acurate, LC Packings) (Schwalbe-Herrmann et al., 2010). Chromatograms can be provided upon request.

HILIC-LC-MS was performed with an internal standard to confirm the ion variations observed in the MS spectra according to the Lipid Maps methods (Ivanova et al., 2007). Amounts of internal standard were added according to the percentage of each PL class in the total PL extract: 7 μg PC (14:0/14:0), 3,5 μg PI (16:0/16:0), 5 μg PE (14:0/14:0), 3,5 μg PA (14:0/14:0) and 3,5 μg CL(14:0/14:0/14:0/14:0), all from Avanti polar lipids. Each molecular lipid species was normalized to the total lipid levels in the corresponding sample. Relative abundance of each ion was calculated by normalizing area of each peak to total area of each class.

Electrospray mass spectrometry conditions

Analysis and interpretation of the MS/MS spectra of each ion identified by LC-MS spectra allowed to identify PL molecular species present in each class as well as their fatty acyl chains composition along the glycerol backbone.

ESI conditions in electrospray linear ion trap mass spectrometer LXQ (ThermoFinnigan, San Jose, CA) were as follows: electrospray voltage was 4.7 kV in negative mode and 5 kV in positive mode; capillary temperature was 275 °C and the sheath gas flow was 25 U. An isolation width of 0.5 Da was used with a 30 ms activation time for MS/MS experiments. Full scan MS spectra and MS/MS spectra were acquired with a 50 ms and 200 ms maximum ionization time, respectively. Normalized collision energy TM (CE) was varied between 17 and 20 (arbitrary units) for MS/MS. Data acquisition of this mass spectrometer was carried out with an Xcalibur data system (V2.0). All the MS spectra obtained were repeated with three independent samples to confirm reproducibility of the results. Relative abundance of each ion was calculated by normalizing area of each peak to total area of each class.

Quantification of hydroperoxides

Hydroperoxides were determined by the FOX2 assay. Aliquots (50 μl) of total lipid extracts were added to FOX2 reagent (950 μl) in a microtube washed with methanol. Standards of H_2O_2 from 0 to 0.8 mM were prepared and underwent the same treatment as the samples. All the mixtures were homogenized in a vortex and incubated for 30 min in the dark at room temperature. The absorbance of samples and standards was read at 560 nm by UV–Vis spectrophotometry.

Statistical analysis

TLC data consisting of three independent experiments performed in triplicate was analyzed using one-way ANOVA and Tukey's multiple post test. Relative ion abundance obtained from three independent cultures by HPLC-MS was analyzed using one-way ANOVA and Tukey's multiple post test. FA relative content quantification was performed from three independent cultures performed in triplicate analyzed by one-way ANOVA and Tukey's multiple post test. Differences were considered significant if $P < 0.05$.

Results and Discussion

Membrane phospholipid profile of rat primary decidual cells was analyzed by thin layer chromatography (TLC) and molecular phospholipid (PL) species were studied by HILIC-LC-MS and MS/MS. Furthermore, a putative association between changes in the cellular PL profile and AEA-induced apoptosis was investigated by comparison of AEA (10 μM, 24 h) treated-group with control group.

We found an increase in total PL content in AEA-treated cells compared to control (about 1.4-fold), suggesting an alteration of lipid synthesis and/or turnover. In this line of evidence, Staff et al. (1999) showed a correlation between an elevated lipid content in decidua basalis with maternal endothelial dysfunction and the pathophysiology of pre-eclampsia.

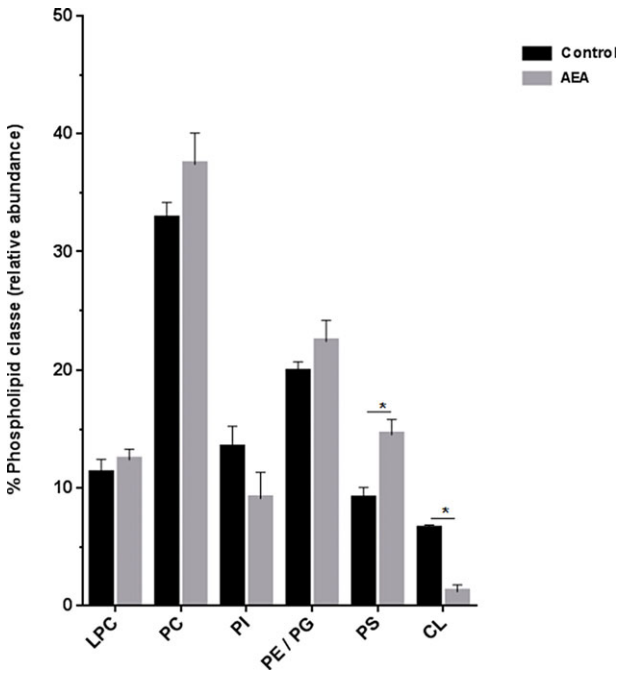

Fig. 1. Relative abundance of phospholipids (PLs) classes in rat decidual cell, in absence and presence of 10 μM anandamide (AEA) for 24 h. PL classes of each group were separated by TLC and phosphate content in each spot was related to total phosphate content in the lipid extract. LPC, lysophosphatidylcholine; PC, phosphatidylcholine, PI, phosphatidylinositol; PE/PG, phosphatidylethanolamine/glycerol; PS, phosphatidylserine CL, cardiolipin. Mean ± SEM values from three independent experiments performed in triplicate are shown (* $P < 0.05$ vs control; one-way ANOVA and Tukey's multiple post test).

A

Fatty Acid	Ratio (T/C)
14:0	1,18
16:1	0,53
16:0	1,07
18:2	0,89
18:1	0,82
18:0	1,16
20:4n6	1,81
20:3n3	1,05
22:6/:5/:4	1,08
22:0	0,57

B

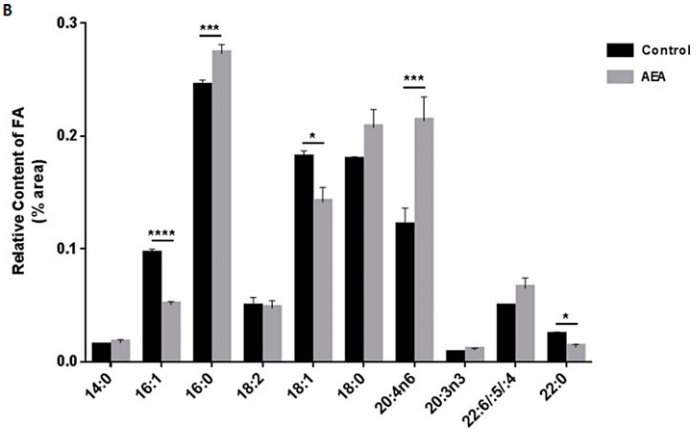

Fig. 2. Gas chromatography quantification of fatty acids in total lipid extract. (A) Fatty acyl profile of total lipid extracts analyzed by GC. Results are shown as ratio of relative content of FA (percent area) from AEA-treated group versus Control group. (B) Analysis of FA substituents of Control vs AEA-treatment. Relative concentrations of fatty acids were calculated by the percent normalized to the sum of all areas of the identified fatty acids. Mean ± SEM values from three independent experiments performed in triplicate are shown (**$P < 0.0001$, ***$P < 0.001$, *$P < 0.05$ p vs control; one-way ANOVA and Tukey's multiple post test). Ratio T/C, Ratio Treatment/Control.**

TABLE 1. Identification of $[M+H]^+$ ions observed in the MS spectra of PC and PE.

	Diacyl					Alkylacyl				
m/z	C:N	FA		FA		m/z	C:N	FA		FA
Phosphatidyl choline										
704.7	30:1	14:1	16:0			718.7	0–32:1	0–16:0	16:1	
706.7	30:0	14:0	16:0			720.7	0–32:0	0–16:0	16:0	
732.7	32:1	16:0	16:1			744.7	0–34:2	0–16:0	18:2	
7347	32:0	16:0	16:0			746.7	0–34:1	0–18:1	16:0	
756.7	34:3	16:1	18:2			748.7	0–34:0	0–18:0	16:0	
758.7	34:2	16:0	18:2	16:1	18:1	766.7	0–36:5	0–16:1	20:4	
760.7	34:1	16:0	18:1			768.7	0–36:4	0–16:0	20:4	
762.7	34:0	16:0	18:0			772.7	0–36:2	0–18:1	18:1	0–16:0 20:2
782.7	36:4	16:0	20:4			774.7	0–36:1	0–18:1	18:0	
784.7	36:3	16:0	20:3			776.7	0–36:0	0–18:0	18:0	
786.7	36:02	18:1	18:1	18:0	18:2	792.7	0–38:6	0–18:2	20:4	
788.7	36:1	18:0	18:1			794.7	0–38:5	0–18:1	20:4	
790.7	36:0	18:0	18:0			796.7	0–38:4	0–18:0	20:4	
804.7	38:7	16:0	22:7							
806.7	38:6	16:0	22:6							
808.7	38:5	18:1	20:4							
810.7	38:4	18:0	20:4							
812.7	38:3	18:0	20:3							
832.7	40:7	18:1	22:6							
834.7	40:6	18:0	22:6	18:1	22:5					
836.7	40:5	18:0	22:5							
Phosphatidyl ethanolamine										
718.5	34:1	16:0	18:1			724.5	0–36:5	0–16:1	20:4	
740.5	36:4	16:0	20:4	18:2	18:2	726.5	0–36:4	0–18:2	18:2	0–16:0 20:4
744.5	36:2	18:2	18:0	18:1	18:1	728.5	0–36:3	18:2	0–18:1	0–18:2 18:1
746.5	36:1	18:0	18:1			750.5	0–38:6	0–18:2	20:4	
748.5	36:0	18:0	18:0			752.5	0–38:5	0–18:1	20:4	0–18:2 20:3
766.5	38:5	18:2	20:3	18:1	20:4	754.5	0–38:4	0–18:1	20:3	
768.5	38:4	18:0	20:4	18:1	20:3	778.5	0–40:6	0–18:0	22:6	
770.5	38:3	18:0	20:3							
772.5	38:2	18:0	20:2	18:2	20:0					
774.5	38:1	18:0	20:1	18:1	20:0					
776.5	38:0	18:0	20:0							
790.5	40:7	18:1	22:6							
792.5	40:6	18:0	22:6	18:1	22:5					
794.5	40:5	18:0	22:5	20:4	20:1					

m/z, mass/charge of each ion; C:N, total number of carbons in the fatty acid chains: total number of double bonds; FA, fatty acids; PC, phosphatydilcholine; PE, phosphatydilethanolamine.
Fatty acid constitution was attributed by the analysis of MS/MS spectra.

TLC of total lipid extracts allowed the identification of distinct spots corresponding to the major phospholipids classes: LPC–lysophosphatidylcholine, PC–phosphatidylcholine, PI–phosphatidylinositol, PS–phosphatidylserine, PE/PG–phosphatidylethanolamine/glycerol, CL–cardiolipin. The relative content (%) of the major PL classes in the total lipid extract remained unchanged (Fig. 1), indicating that the increase in total PL levels were not caused by an increment of synthesis of a single class but due to the modification of precursors levels, or a general increase in phospholipid synthesis.

PC was the dominant phospholipid component followed by PE and PS in decidual cells. PS content increased in the treated group, which might be reflective of the induced AEA-apoptotic cell death. Although generally considered as a result of alterations in the phospholipid asymmetry, a more complex machinery may be involved. In support of these data, it has been suggested that increased PS outward movement further enhanced PS de novo biosynthesis (Yu et al., 2004; Ferrara et al., 2013). In fact, a lipidomic analysis of human placental syncytiotrophoblast microvesicles associated high PS levels with activation of apoptosis in pre-eclampsia (Baig et al., 2013).

In parallel, we found a significant decline in CL levels in treated group, which may underlie mitochondrial dysfunction. We have previously reported that AEA-induced apoptosis was associated with ceramide accumulation, mitochondrial generated reactive oxygen species (ROS) and caspase 3/7 activation (Fonseca et al., 2013b). Among the factors associating CL changes and apoptosis, acyl chain remodeling and CL oxidation associated with ROS generation were reported (Petrosillo et al., 2001; Schug and Gottlieb, 2009). Moreover, decreased CL synthesis and cytochrome *c* release into the cytosol, were critical causes in a model palmitate-induced cardiomyocyte apoptosis (Ostrander et al., 2001). On the other hand, a decrease in CL has been related with ceramide production through the reverse activity of a mitochondrial-membrane ceramidase in rat brain (El Bawab et al., 2001). Nevertheless, CL oxidation products have been described as apoptotic mediators and a potential cause for the decrease of CL content observed in apoptosis (Tyurina et al., 2006).

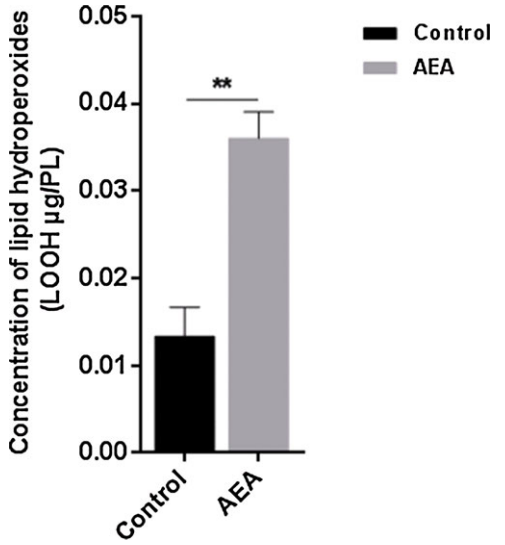

Fig. 3. Concentration of lipid hydroperoxides - Concentration of lipid hydroperoxides (LOOH µg/PL) in the total lipid extract in Control group and AEA-treated group evaluated by FOX II assay. Mean ± SEM values from three independent experiments performed in triplicate are shown ($P < 0.01$ vs control; one-way ANOVA and Tukey's multiple post test).**

To further investigate the effects of AEA on membrane structure profile, we quantified the total fatty acids by GC-FID (Fig. 2). Considerable changes were noticed, in particular an increase in the relative content of long chain fatty acids (FA) with high degree of unsaturation. Fluidity properties of biological membranes are decisive for cell homeostasis and networks of signal-transduction pathways. Membranes containing shorter-chain lipids and polyunsaturared fatty acids (PUFA) are more fluid (Catala, 2012). Hence, our results may suggest a possible protective/compensation mechanism against changes in physical properties.

Phospholipids containing PUFAs are of fundamental significance for a large number of biological functions (Chaurio et al., 2009). The high degree of polyunsaturation may confer distinct lipid bilayer properties that are, for example, crucial for activation and stability/function of some intrinsic membrane proteins, like G protein-coupled receptors (GPCR) (Rajamoorthi et al., 2005). This fact is of particular importance because AEA induces decidual cell apoptosis through cannabinoid receptor I (CB1), a GPCR. On the other hand, we previously described that higher AEA levels, due to its lipophilic nature, exerts cytotoxic effects on rat decidual cells, probably by interacting with cell membrane (Fonseca et al., 2009). Moreover, lipid composition is also expected to play an important role in modulating the integral membrane FAAH enzyme availability and activity.

The analysis of total FA (Fig. 2), showed an increase of FA (20:4n6), which corresponds to arachidonic acid (AA). The fact that AA is a direct product of AEA hydrolysis by intracellular FAAH can sustain for the increase observed in treated group. Besides regulating apoptosis itself (Cao et al., 2000; Pompeia et al., 2003) AA is a precursor of important lipid mediators, PGs and eCBs. Therefore, the enrichment of AA content in AEA-treated group may disturb eCBs and PGs, both metabolic and signaling pathways. On the other hand, AA-containing phospholipid bilayers are disordered and deformable (Rajamoorthi et al., 2005).

TABLE 2. Identification of $[M+H]^-$ ions observed in the MS spectra of PS and PI.

	Diacyl					Alkylacyl					
m/z	C:N	FA		FA		m/z	C:N	FA		FA	
Phosphatidylserine											
758.4	34:2	16:0	18:2	16:1	18:1	716.48	o–32:2	o-16:1	16:1		
760.4	34:1	16:0	18:1	16:1	18:0	744.46	o–34:2	o-16:1	18:1		
782.4	36:4	16:0	20:4								
786.4	36:2	18:0	18:2	18:1	18:1						
788.4	36:1	18:0	18:1								
810.4	38:4	18:0	20:4								
812.4	38:3	18:0	20:3	18:1	20:3						
834.4	40:6	18:0	22:6								
Phosphatidylinositol											
833,6	34:2	16:1	18:1	16:0	18:2	817,6	o–34:3	o–16:1	18:2		
835,6	34:1	16:0	18:1	16:1	18:0	819,6	o–34:2	o–16:1	18:1		
857.6	36:4	16:0	20:4			821,6	o–34:1	o–16:0	18:1		
861,6	36:2	18:1	18:1	18:2	18:0	843,6	o–36:4	o–16:0	20:4	o–16:1	20:3
865,6	36:0	18:0	18:0								
883.6	38:5	18:1	20:4	20:3	18:2						
885.6	38:4	18:0	20:4	18:1	20:3						
887,6	38:3	18:0	20:3								

m/z, mass/charge of each ion; C:N, total number of carbons in the fatty acid chain: total number of double bonds; FA, fatty acids; PS, phosphatydilserine; PI, phosphatidylinositol. Fatty acid constitution was attributed by the analysis of MS/MS spectra.

Underlying PL metabolism, it is known that saturated fatty acids (SFA) are successively transformed by the action of elongases into palmitic acid (16:0), which is then elongated to stearic acid (18:0), and other long chain SFAs. Interestingly, the relative increase of FA (18:0) was accompanied by a decrease in ratio of saturated to unsaturated FA with treatment (Table 1). Also, FA (18:2) serve as a substrate to be converted into longer fatty acids, as arachidonic acid (C20:4) and docosahexaenoic acid (C22:6/:5/:4), via a series of oxidative desaturation and elongation reactions (Jump, 2002). The latter, plays important roles on membrane fluidity, signaling, apoptosis and gene expression. Together with a relative decrease in FA (18:2) and FA (18:1), we found a significant increase in PUFAs (Fig. 2).

Unsaturated fatty acids are prominent target of oxidative attack that can cause cellular dysfunction via different mechanisms (Prasad et al., 2010). Moreover, lipid peroxidation process can disturb the membrane assembly, causing changes in fluidity and permeability, modifications of ion transport and inhibition of metabolic processes (Nigam and Schewe, 2000). Consequently, the increase of PUFA by AEA treatment may favor oxidative reactions, producing oxidation products with biological activities. On the other hand, the conformational shift in fatty acyl chain upon oxidation may trigger numerous downstream pathways, as apoptosis (Catala, 2012). In order to confirm the presence of lipid oxidation, the quantification of lipid hydroperoxides was performed using a modified ferrous oxidation-xylenol orange (FOX II) assay. The results showed an increase in the lipid hydroperoxides content in the treated group in comparison with control (Fig. 3), which corroborates to the putative phospholipid oxidation and the increase in reactive-oxygen species in our model of AEA induced-apoptosis (Fonseca et al., 2013b).

Profiling of PL molecular species

To achieve the PL profile at the molecular level, HILIC-MS and MS/MS was performed in positive and negative modes. PC, PE, and LPC were identified by the analysis in positive mode (Table 1) while PE, PS, PI and CL were identified in negative mode (Table 2). This approach allowed the identification of fatty acyl distribution in each PL class. The low abundance of LPC, SM and CL did not allowed further analysis, although the main molecular species were identified (Table 3; Supplementary material).

A

	m/z	C:N	Ratio T/C
Phosphatidylcholine	704.7	30:1	0.52 ***
	706.7	30:0	0.71
	718.7	o-32:1	1.11
	720.7	o-32:0	1.08
	732.7	32:1	1.06
	7347	32:0	1.17
	744.7	o-34:2	0.59 ***
	746.7	o-34:1	0.83
	748.7	o-34:0	0.96
	756.7	34:3	1.15
	758.7	34:2	0.91
	760.7	34:1	0.89
	762.7	34:0	0.92
	766.7	o-36:5	1.45 ***
	768.7	o-36:4	1.22
	772.7	o-36:2	0.73
	774.7	o-36:1	0.79
	776.7	o-36:0	1.35 ***
	782.7	36:4	1.75 ****
	784.7	36:3	1.09
	786.7	36:02	0.74
	788.7	36:1	0.86
	790.7	36:0	1.16
	792.7	o-38:6	1.71 ***
	794.7	o-38:5	1.08
	796.7	o-38:4	1.96 ****
	804.7	38:7	1.52
	806.7	38:6	1.24
	808.7	38:5	1.10
	810.7	38:4	1.62 ***
	812.7	38:3	1.13
	832.7	40:7	1.32
	834.7	40:6	1.03
	836.7	40:5	1.25

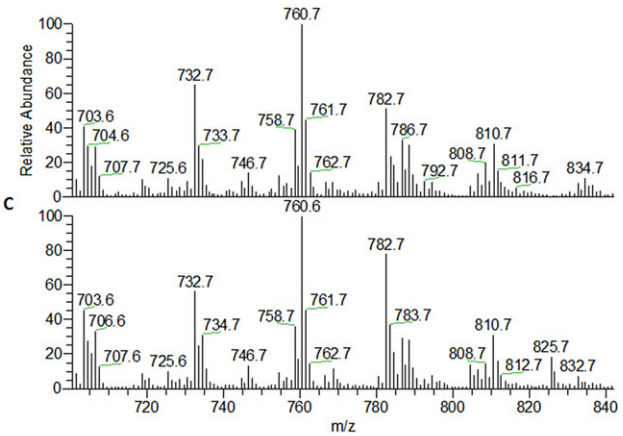

Fig. 4. Analysis of PCs molecular species profile changes and MS spectra. (A) Analysis of PCs molecular species changes expressed as a ratio of relative content of Treated group vs Control group. (B) HILIC-MS and MS/MS spectra for PCs in the positive mode $[M\text{-}H]^+$ of Control group. (C) HILIC-MS and MS/MS spectra for PCs in the positive mode $[M\text{-}H]^+$ of AEA-treated group. Significant differences between groups were evaluated. Results from three independent experiments are shown (** $P < 0.0001$, *** $P < 0.001$ vs control; Multiple t test, and Sidak-Bonferroni post test). m/z, mass/charge of each ion; C:N, total number of carbons in the fatty acid chains: total number of double bonds; o-C:N, plasmalogens; Ratio T/C, Ratio Treatment/Control.**

Phosphatidylcholine

Phosphatidylcholine (PC) is the major structural phospholipid component of eukaryotic membranes and is mostly located in the outer leaflet and contributes to both growth and programmed cell death (Ridgway, 2013). Analysis of molecular species (Table 1, Fig. 4) revealed that PC (16:0/18:1) and PC (16:0/20:4) corresponding to $[MH]^+$ at *m/z* 760.7 and 782.7 were the most relative abundant, followed by PC (16:0/18:2), PC (18:1/18:1), PC (16:0/16:1), PC (o-18:0/18:0) and PC (18:0/20:4) at *m/z* 758.7, 786.7, 732.7, 776.7 and 810.7, respectively. The less relatively abundant PCs were alkylacyl PCs, as PC (o-16:0/16:0, o-18:0/16:0, o-18:1/18:0 (18:1)) corresponding to ions at *m/z* 720.7, 748.7 and 774.7 respectively. Diacyl PCs species are more abundant than alkylacyl PC in rat decidual cells. PCs (o-36:0; o-36:5; 36:4; o-38:6; o-38:4; 38:7 and 38:4), correspondent to the PC molecular species bearing longer FA chain, were the species that exhibited the higher relative content increase in the treated group. These species contain the FA (20:4), assigned to AA.

Phosphatidylethanolamine

Ethanolamine phospholipids (PE) are key membrane fluidizing PLs. Moreover they have also been linked to apoptosis (Emoto et al., 1997). PE was analyzed by ESI–MS in positive mode, with formation of $[M + H]^+$ (Table 1, Fig. 5). The diacyl PE (18:0/20:4) corresponding to $[M + H]^+$ at *m/z* 768.5 was the higher relative abundant molecular specie, followed by alkylacyl PEs (o-18:2/20:4; o-18:1/20:4; o-16:1/20:4) at *m/z* 752.7; 750.5 and 724.5, respectively, and PE (18:0/18:1) at *m/z* 748.6. Similarly to results obtained for PC, there is a slight increase in PE with longer FA chains.

PC and PE plasmalogens

Plasmalogens (Pls) comprise all plasmenyl and plasmanyl phospholipids containing ethanolamine or choline in the head group of the glycerol backbone, synthesized in peroxisomes. Besides structural functions, Pls have been implicated in several biological processes where they can affect membrane fluidity, mediate signal transduction, protect against oxidative stress and modulate pathophysiological changes (Brites et al., 2009; Wanders and Waterham, 2006). Several studies have supported the hypothesis that plasmalogens, due to their high susceptibility to oxidative damage, possess antioxidant properties (Khaselev and Murphy, 2000; Lessig and Fuchs, 2009). In this study we found that decidual cells exhibit high abundance of plasmalogens, and also, we found an significant increase of relative content the of plasmenyl PCs (o-16:1/20:4; o-18:2/20:4) and plasmanyl PCs (o-18:0/18:0; o-18:0/20:4) molecular species with treatment (Fig. 4). This fact is of particular relevance, as it has been demonstrated that upon decidualization, endometrial stromal cells became resistant to

A

	m/z	C:N	Ratio T/C
Phosphatidylethanolamine	718.5	34:1	0.95
	724.5	o-36:5	1.04
	726.5	o-36:4	1.09
	728.5	o-36:3	0.74 ***
	740.5	36:4	1.41
	744.5	36:2	0.87
	746.5	36:1	0.98
	748.5	36:0	0.77 ****
	750.5	o-38:6	1.07
	752.5	o-38:5	1.10
	754.5	o-38:4	1.15
	766.5	38:5	0.90
	768.5	38:4	1.12
	770.5	38:3	1.00
	772.5	38:2	1.12
	774.5	38:1	1.00
	776.5	38:0	1.20
	778.5	o-40:6	0.82
	790.5	40:7	1.73 ***
	792.5	40:6	0.92
	794.5	40:5	1.30

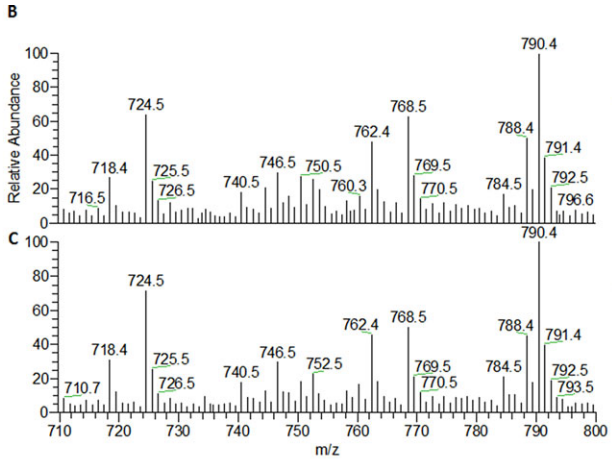

Fig. 5. Analysis of PEs molecular species profile changes and MS spectra. (A) Analysis of PEs molecular species changes expressed as a ratio of relative content of Treated group vs Control group. (B) HILIC-MS and MS/MS spectra for PEs in the positive mode $[M-H]^+$ of Control group. C) HILIC-MS and MS/MS spectra for PEs in the positive mode $[M-H]^+$ of AEA-treated group. Significant differences between groups were evaluated. Results from three independent experiments are shown (** $P < 0.0001$, *** $P < 0.001$ vs control; Multiple *t* test, and Sidak-Bonferroni post test). m/z, mass/charge of each ion; C:N, total number of carbons in the fatty acid chains: total number of double bonds; o-C:N, plasmalogens; Ratio T/C, Ratio Treatment/Control.**

oxidative stress (Kajihara et al., 2013). In addition, a recent study suggested an anti-apoptotic action of PIs in the brain by enhanced phosphorylation of the phosphoinositide 3-kinase (PI3K)-dependent serine/threonine-specific protein kinase Akt and extracellular-signal-regulated kinases ERK1/2 (Hossain et al., 2013). Although, contradictory studies reported that plasmalogen-containing cell membranes are less fluid, more instable and susceptible to oxidative damage than the plasmalogen-deficient membranes (Hermetter et al., 1989). Our results may indicate that plasmalogens may provide a robust defensive mechanism of decidual cells against oxidative stress. Moreover, upon AEA-treatment rat decidual cells may reinforce this response to oxidative stress, by synthesizing plasmalogens, though these findings require further clarifications.

Phosphatidylserine

Phosphatidylserine (PS) is a negatively charged PL, normally located in inner membrane leaflet. PS was analyzed by ESI–MS

A

	m/z	C:N	Ratio T/C
Phosphatidylserine	758.4	34:2	1.19
	760.4	34:1	1.03
	782.4	36:4	2.03 ****
	786.4	36:2	0.95
	788.4	36:1	0.85 *
	810.4	38:4	1.24 ****
	812.4	38:3	1.06
	834.4	40:6	1.10

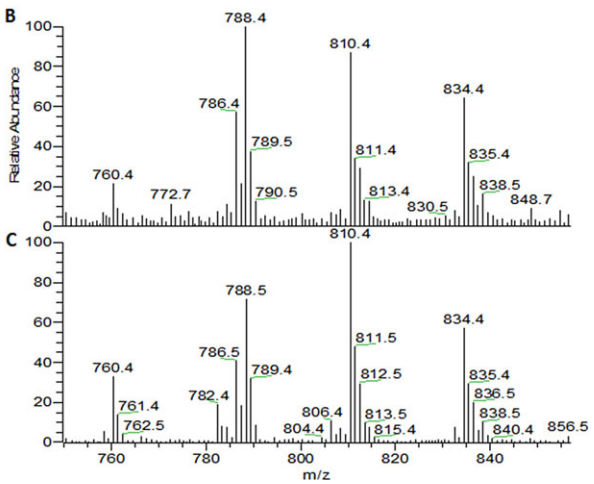

Fig. 6. Analysis of PSs molecular species profile changes and MS spectra. (A) Analysis of PSs molecular species changes expressed as a ratio of relative content of Treated group vs Control group. (B) HILIC-MS and MS/MS spectra for PSs in the negative mode $[M-H]^-$ of Control group. (C) HILIC-MS and MS/MS spectra for PSs in the negative mode $[M-H]^-$ of AEA-treated group. Significant differences between groups were evaluated. Results from three independent experiments are shown (** $P < 0.0001$, * $P < 0.05$ vs control; Multiple *t* test, and Sidak-Bonferroni post test). m/z, mass/charge of each ion; C:N, total number of carbons in the fatty acid chain: total number of double bonds; o-C:N, plasmalogens; Ratio T/C, Ratio Treatment/Control.**

A

	m/z	C:N	Ratio T/C
Phosphatidylinositol	817.6	o-34:3	0.78
	819.6	o-34:2	0.60
	821.6	o-34:1	0.78
	833.6	34:2	0.89
	835.6	34:1	0.77
	843.6	o-36:4	0.85
	857.6	36:4	1.00
	861.6	36:2	0.78
	865.6	36:0	0.50*
	883.6	38:4	0.91
	885.6	38:3	1.08
	887.6	38:3	1.01

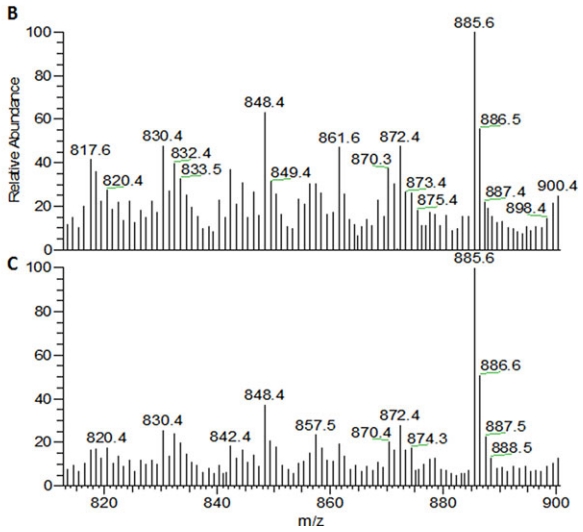

Fig. 7. Analysis of PIs molecular species profile changes and MS spectra. (A) Analysis of PIs molecular species changes expressed as a ratio of relative content of Treated group vs Control group. (B) HILIC-MS and MS/MS spectra for PIs in the negative mode [M-H]⁻ of Control group. (C) HILIC-MS and MS/MS spectra for PIs in the negative mode [M-H]⁻ of AEA-treated group. Significant differences between groups were evaluated. Results from three independent experiments are shown (* $P < 0.05$ vs control; Multiple *t* test, and Sidak-Bonferroni post test). m/z, mass/charge of each ion; C:N, total number of carbons in the fatty acid chains: total number of double bonds; o-C:N, plasmalogens; Ratio T/C, Ratio Treatment/Control.

in negative mode, with formation of $[M–H]^-$ (Table 2, Fig. 6). The species with highest relative abundance identified were PS (18:0/18:1), PS (18:0/20:4), and PS (18:0/22:6) corresponding to the ion $[M–H]^-$ at m/z of 788.4, 810.4 and 834.4. PS (16:/20:4) and PS (18:0/20:4) at *m*/*z* 782.4 and 810.4 were the species which exhibited a higher relative increase and just PS (18:0/18:1) at *m*/*z* 788.4 decreased with AEA-treatment.

PS exposure on the external leaflet of the plasma membrane is widely observed during early apoptosis (Vance and Steenbergen, 2005). Despite, it has also been suggested that over expression of PS synthases and accumulation of PS might enroll a protective effect against apoptosis in different systems (Yu et al., 2004; Kim et al., 2010; Ferrara et al., 2013). As with results obtained for TLC our findings suggest that PS is being synthesized during the AEA-apoptotic stimuli.

Phosphatidylnositol

Phosphatidylinositol (PI) was analyzed by ESI–MS in negative mode, with formation of [M-H]⁻ ions (Table 2, Fig. 7). PI (18:0/20:4) corresponding to *m*/*z* 885.6 was the most relative abundant, followed by PI (18:0/20:3) at *m*/*z* 887.6 and PI (16:0/20:4) at *m*/*z* 857.6.

PI is the primary source of the AA required for eicosanoids biosynthesis by enzyme phospholipase A2 (PLA2). It was observed a decreased of the majority of PI species. PIs display major roles in intracellular signaling and cell proliferation/apoptosis, and its downregulation may represent a protective response against proapoptotic stressors. Furthermore, phosphoinositides like phosphatidylinositol 3,4,5-trisphosphate (PIP3) and phosphatidylinositol 3,4-biphosphate (PIP2) promote cell survival and protect against apoptosis by activating Akt/PKB phosphorylating components of the apoptotic machinery. Also inhibition of phosphatidylinositol 3-Kinase-Akt signaling blocked growth, and promoted apoptosis in lung cells (Krystal et al., 2002).

As aforementioned we observed an increase in PS levels. Both PS and PI share the same substrate (CDP-diacylglycerol or CDP-DAG) for synthesis. This may imply that probably AEA displayed a preference on up-regulation of PS synthesis over PI synthesis, probably by downregulation of PI synthesizing enzyme.

Conclusion

Membrane is strictly and dynamically regulated. Increased phospholipid metabolism involves a multifactorial over-activation of phospholipases and elongases, imbalances in saturated/unsaturated fatty acids levels and oxidative stress. As a result it may disrupt the framework of membrane dynamics and have further harmful biologic effects. We described here a possible key role of phospholipid changes in mediating AEA effects, although it may also embody a natural consequence of the membrane alterations associated with apoptosis. Albeit, in overall our data brings out a possible adaptation, or rather a consequence of membrane metabolism in response to AEA-induced cell death by essentially two mechanisms: fatty acid desaturation pathway and the deacylation-reacylation cycle, resulting in a reorganization of membrane phospholipidome and biochemical properties. Altered cellular lipid metabolism contributes to pregnancy disorders and hence changes in the lipidome may reflect metabolic alterations. To our knowledge, this is the first study considering AEA effect on rat decidua remodeling, revealing compositional changes in phospholipid molecular species, and may provide a global vision of an adaptive response of this tissue to apoptotic stimuli. On the other hand, membrane dynamics and integrity changes may also affect endocannabinoid signaling and uptake. Deregulation of the decidualization process may hamper the success of pregnancy. Therefore, in the future, lipidomic approach may provide a powerful tool to better understand the mechanisms underlying the uterine remodeling process and the impact of endocannabinoid system disturbance on decidual phospholipid metabolism during pregnancy..

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Manuscript II:

Anandamide and decidual remodelling: COX-2 oxidative metabolism as a key regulator

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Anandamide and decidual remodelling: COX-2 oxidative metabolism as a key regulator

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ABSTRACT

Recently, endocannabinoids have emerged as signalling mediators in reproduction. It is widely accepted that anandamide (AEA) levels must be tightly regulated, and that a disturbance in AEA levels may impact decidual stability and regression. We have previously characterized the endocannabinoid machinery in rat decidual tissue and reported the pro-apoptotic action of AEA on rat decidual cells. Cyclooxygenase-2 (COX-2) is an inducible enzyme that plays a crucial role in early pregnancy, and is also a key modulator in the crosstalk between endocannabinoids and prostaglandins. On the other hand, AEA-oxidative metabolism by COX-2 is not merely a mean to inactivate its action, but it yields the formation of a new class of mediators, named prostaglandin-ethanolamides, or prostamides. In this study we found that AEA-induced apoptosis in decidual cells involves COX-2 metabolic pathway. AEA induced COX-2 expression through p38 MAPK, resulting in the formation of prostamide E2 (PME2). Our findings also suggest that AEA-induced effect is associated with NF-kB activation. Finally, we describe the involvement of PME2 in the induction of the intrinsic apoptotic pathway in rat decidual cells. Altogether, our findings highlight the role of COX-2 as a gatekeeper in the uterine environment and clarify the impact of the deregulation of AEA levels on the decidual remodelling process.

1. Introduction

The cyclical waves of proliferation, differentiation, shedding, and regeneration of the endometrium are a remarkable feature during the reproductive life. The endometrial stromal cells proliferate and differentiate into specialized decidual cells in a process named decidualization. In humans, this process represents a tipping point that ensures, every month, the achievement of a receptive uterus primed for implantation [1], while in rodents the decidual reaction occurs in response to blastocyst implantation [2]. Although the highly dynamic nature of the endometrium is well documented, the mechanisms that orchestrate this process are poorly clarified.

Over the last decades, the upcoming role of endocannabinoids as signalling mediators emerged in several physiological and pathophysiological functions. The endocannabinoid system (ECS) comprises two G-protein-coupled cannabinoid receptors (CB1 and CB2), their endogenous ligands (the endocannabinoids, eCBs), and biosynthetic and hydrolysing enzymes for eCBs [3]. This is an evolving system, operating in coordination with a hormonal and cytokine network, which controls several reproductive events, including decidualization [3].

Anandamide (AEA) is one of the main endocannabinoids. A physiological tone of AEA, that warrants a receptive uterine environment, is mainly mediated by N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), the enzyme responsible for AEA synthesis, and fatty acid amide hydrolase (FAAH), the enzyme in charge of AEA degradation into ethanolamide and arachidonic acid (AA). The latter serves as a precursor for prostaglandin synthesis by cyclooxygenase-2 (COX-2) [4]. In mice and humans, a shift in the balance between AEA levels and FAAH activity has been correlated with impaired implantation and/or miscarriages [5]. Besides hydrolyses by FAAH, AEA may undergo direct oxygenation by COX-2, resulting in the formation of prostaglandin-ethanolamides, also termed prostamides (PMs), such as PME2 and PMF2α [6]. The oxidative metabolism of AEA, initially considered an alternative catabolic route, has recently gained relevance as a source of new lipid mediators, with unique biological activity [7]. It has been described that COX-2 mediates several cellular events of eCBs, and that AEA modulates PG production and COX-2 expression in reproductive tissues and other systems [7–10]. For instance, COX-2 is involved in the AEA-induced apoptosis in human cervical carcinoma cells and lung cancer cells [11–13]. It is known that COX-2 is critical in implantation and decidualization [14], though the underlying mechanisms are still unknown. Moreover, although several lines of evidence reinforce the interplay between endocannabinoids and prostaglandins, the intricate crosstalk is not yet clarified.

Apoptosis in the endometrium plays an essential role for endometrial receptivity and early pregnancy. Previously, the pro-apoptotic-effect of AEA in rat decidual cells has been described [15], unravelling the

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central role of this lipid mediator in decidual turnover. In the current study we investigated the role of COX-2 oxidative metabolism of AEA in its pro-apoptotic action in rat primary decidual cells, and the molecular mechanisms that underlie COX-2 regulation. We hypothesize that a disruption of FAAH activity, and/or aberrant eCB signalling, may favour COX-2-mediated oxidation of AEA and overwhelm the decidual regression process through the formation of pro-apoptotic PMs, thereby impairing the pregnancy outcome.

2. Material and methods

2.1. Animals

All animal experiments were conducted with Direção-Geral de Alimentação e Veterinária (DGAV) approval and conformed to the European legislation on the use of laboratory animals. Briefly, female Wistar rats (Charles River Laboratories, Barcelona, Spain) were mated and the day on which spermatozoa were found in the morning vaginal smear was designated as day 1 of pregnancy. On day 10 of pregnancy, animals were anesthetised and sacrificed and uterine horns were collected for cell culture studies.

2.2. Cell cultures

Mesometrial decidua was dissected from antimesometrial decidua and digested with dispase II (2.4 U/ml) (Roche Diagnostics GmbH, Mannheim, Germany), colagenase (50 U/ml) (Sigma Chemical Co, St. Louis, MO, USA) and Dnase I (200 U/ml) (Roche Diagnostics GmbH, Mannheim, Germany) in HBSS (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) for 1 h at 37 °C. Non-digested tissue was removed by filtration through a 60 μm mesh and decidual cells were collected by centrifugation at 200 *g* for 10 min as previously described [19]. Erythrocytes were removed by the addition of NH4Cl solution (0.83%) followed by incubation on ice for 10 min. Cells were cultured in Dulbecco's minimum essential medium (DMEM) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) supplemented with antibiotic–antimicotic solution (200 U/ml penicillin G, 0.5 μg/ml amphotericine B and 200 μg/ml streptomycin) (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and 10% FBS (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) under a 95% air–5% CO_2 humidified atmosphere for 4 h. After adhesion, cells were cultured for 24 h in DMEM without FBS in the presence or absence of anandamide (AEA, 10 μM). In order to study the involvement of COX-2, cells were pre-incubated with selective COX-2 inhibitors Celecoxib (0.1 μM) and R-Flurbirpofen (1 μM), 30 min before AEA treatment. The effects of AEA in the presence of a FAAH inhibitor, URB 597 (10 μM), added 30 min previously to AEA treatment were studied. The inhibitors of p38 MAPK (SB202190; 25 μM) and p44/42 (PD98059; 20 μM), were added 30 min before AEA. Stock solutions were prepared in ethanol and stored in aliquots at −80 °C. For mesometrial explant cultures, 100 mg of mesometrial decidua was cut out and pinned in sterile plastic organ-culture dishes and maintained at same conditions as the primary decidual cell cultures. After treatment the decidual fragment and medium were collected and stored at −80 °C.

2.3. Immunocytochemistry

Cells (2.5×10^5 cells/well) were seeded on eight-well glass chamber slides and incubated for 24 h in the presence or absence of anandamide. After incubation, cells were fixed for 15 min at room temperature in 4% paraphormaldehyde. After fixation, cells were permeabilized with PBS, 0.1% Triton X-100 and 0.2% Tween-20 solution. Nuclear factor-kappaB (NF-kB) expression, was analysed using an avidin–biotin alkaline phosphatase complex immunocytochemical technique (Vectastain ABC kit, Vector Laboratories, CA, USA). The slides were incubated overnight at 4 °C with the primary rabbit NF-kB (1:100) (sc-372, Santa Cruz, CA, USA). After washing with PBS they were incubated with diluted biotinylated secondary antibody for 30 min, followed by incubation with Vectastain ABC-AP reagent as recommended in the kit instructions. The reaction was developed by incubation with Sigma Fast Red™ tablets (Sigma® FastTM Fast Red-TR/Naphtol). Negative controls were performed with the inclusion of rabbit or goat IgG instead of the primary antibody. The slides were counterstained with Mayer's Haematoxylin solution (Sigma Chemical Co, St. Louis, USA) and mounted in Aquamont improved medium (BDH Laboratory Supplies, Pool, England).

2.4. Cell viability, cytotoxicity assays and nuclear morphology

Cells were cultured in ninety-six well plates at a density of 2.5×10^5/ml for 12–48 h in the presence or absence of AEA (0.1–25 μM). After incubation, MTT (0.5 mg/ml final concentration) (Sigma Chemical Co, St. Louis, MO, USA) was added and the plate was incubated for 3 h at 37 °C. The formazan was quantified spectrophotometrically by the addition of DMSO:isopropanol mixture (3:1). LDH release was measured using CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. Control experiments with equimolar concentrations of ethanol or DMSO alone or with the inhibitors did not show any significant effect on the parameters investigated in this study (data not shown). The results of cell viability are the mean of three independent experiments performed in triplicate and are expressed as a percentage of the untreated control cells. The alterations in cell morphology induced by PME2 (10 μM) treatment for 24 h were analysed by Höechst staining. This concentration was selected according to the viability assays of the cellular model. After treatment, cells plated in 24-well lamellae were fixed with methanol, stained (0.5 mg/ml Höechst 33342) and and examined under a fluorescence microscope (Eclipse E400, Nikon, Japan) equipped with an excitation filter with maximum transmission at 360/40 nm.

2.5. Evaluation of mitochondrial membrane potential ($\Delta\psi m$) and intracellular reactive oxygen species (ROS)

For the assessment of $\Delta\psi m$ and ROS production, decidual cells were seeded in 96-well black plates and treated for 24 h. For $\Delta\psi m$ studies, cells were washed and incubated with DiOC6 40 nM, for 15 min, at 37 °C, in the dark. For evaluation of ROS production, cells were incubated with the fluorescent probe 2,7-Dichlorodihydrofluorescein-diacetate (DCDHF-DA), for 1 h, at room temperature. The fluorescence intensity was detected using a Microplate Fluorimeter (BioTek Instruments, Vermont, USA), with excitation and emission wavelengths of 488 nm and 525 nm, respectively. The positive controls for $\Delta\psi m$ or ROS production were the carbonyl cyanide m-chlorophenyl hydrazone (CCCP) or phorbol 12-myristate 13-acetate (PMA), respectively. The results were expressed in relative fluorescence units (RFU). For all measurements, basal fluorescence was subtracted.

2.6. Determination of caspase-3/-7 and -9 activities

The caspase-GloTM-3/-7 and -9 assays (Promega Corporation, Madison, WI, USA) were used for the evaluation of caspase-3/-7 and -9 activities. Cells were seeded (2×10^4/well) in 96-well microplates, for 18 h and the respective caspase reagents were added to the cells according to kit instructions Luminometer readings were taken after 1.5 h of incubation at room temperature. Staurosporine (STS) was used as a positive control. The resultant luminescence was measured in relative light units (RLU) using a Microplate Luminometer (BioTek Instruments, Vermont, USA).

2.7. Western blotting

Western blotting was performed for analysis of COX-2, MAPK kinase and IkB-α protein levels. Decidual cells were cultured and treated with AEA for 1 h, 6 h and 24 h, in the presence and absence of URB 597. Cell

extracts were prepared in potassium phosphate buffer containing a cocktail of protease and phosphatase inhibitors. Protein concentrations were measured by Bradford assay. Samples (25 μg) were run on 10 or 12.5% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were then incubated with antibodies against goat-COX-2 (sc-1745, Santa Cruz Biotechnology), rabbit Ikβ-α (sc-847, Santa Cruz Biotechnology) and rabbit phospho-p38 Thr180/Tyr182 (9215), phospho-p44/42 Thr202/Tyr204 (9101), phospho-JNK (Thr183/Tyr185, 9251) (Cell Signaling Technology) or the respective non-phosphorylated forms (anti-human rabbit IgG, Cell Signaling Technology) at 4 °C overnight. Membranes were then washed and incubated with goat or rabbit IgG horseradish peroxidase-conjugated antibody, and detected by enhanced chemiluminescence. β-Tubulin (H-235, Santa Cruz Biotech) was used as a loading control. To quantify and compare protein levels, the density of each band was measured by densitometry.

2.8. COX-2 enzyme activity assay

After treatments, cells were collected by centrifugation. Cell pellets were lysed in cold buffer (0.1 M Tris–HCl, pH 7·8 containing 1 Mm ethylenediaminetetraacetic acid) and centrifuged at 10,000 *g* for 15 min at 4 °C. The COX-2 activity was measured by the peroxidase activity of COX-2. Briefly, the peroxidase activity was assayed colorimetrically by monitoring the appearance of oxidized N,N,N′,N′-tetramethyl-p-phenylenediamine (TMPD) at 590 nm. The assay mixture containing assay buffer, heme and sample, were incubated for 5 min at 25 °C, and TMPD was added. The reactions were initiated by adding arachidonic acid and incubated for 5 min at 25 °C and the absorbance read at 590 nm. COX-2 activity was then calculated using the following formula whereby 1 unit is defined as the amount of enzyme to oxidize 1 nmol of TMPD per min at 25 °C: COX-2 activity = $[(\Delta 590 / 5\ \text{min} / 0.00826\ \mu M - 1) \times (0.21\ \text{ml} / 0.01\ \text{ml})] / 2$.

2.9. Extraction of COX-2-derivatives and development of an LC–MS method for their quantification

Extraction from cell pellet and tissues, purification and quantification of AEA, 2-AG and their COX-2-derived metabolites such as PMs and prostaglandin-glycerol esters (PG-GEs), such as PMF2α, PME2, PGF2α-GE and PGE2-GE, require several biochemical steps [16]. First, samples are homogenized and extracted with acetone containing internal deuterated standards (i.e. [2H]8AEA, [2H]52AG, [2H]4PMF2α, [2H]4PME2, [2H]4 PGF2α-GE and [2H]4 PGE2-GE) for AEA, 2-AG, PMF2α, PME2, PGF2α-GE and PGE2-GE quantification by isotope dilution LC–MS. The lipid-containing organic phase is dried down, weighed and pre-purified by open bed chromatography on silica gel. Fractions are obtained by eluting the column with 99:1, 90:10, 70:30 and 50:50 (v/v) chloroform/methanol. The 90:10 fraction is used for AEA and 2-AG quantification by liquid chromatography–atmospheric pressure chemical ionization-mass spectrometry (LC–APCI-MS), using selected ion monitoring at M^{+1} values for the four compounds and their deuterated homologues. The 70:30 fraction is used for COX-2 derivatives quantification by LC–MS-IT-TOF analysis (Shimadzu Corporation, Kyoto, Japan) equipped with an ESI interface, using multiple reaction monitoring. The chromatograms of the high-resolution $M^{+}Na^{+}$ values are extracted and used for calibration and quantification. AEA and 2-AG were measured as previously described [17]. COX-2 derivatives were measured by LC–MS–MS, using an LC20AB coupled to a hybrid detector IT-TOF (Shimadzu Corporation, Kyoto, Japan) equipped with an ESI interface. LC analysis was performed in the isocratic mode using a Discovery® C18 column (15 cm × 2.1 mm, 5 μm) and methanol/water/acetic acid (53:47:0.05 by vol.) as mobile phase with a flow rate of 0.15 ml/min. Identification of PME2, PMF2α, PGE2-GE and PGF2α-GE was carried out using ESI ionization in the positive mode with nebulizing gas flow of 1.5 ml/min and curved dessolvation line temperature of 250 °C.

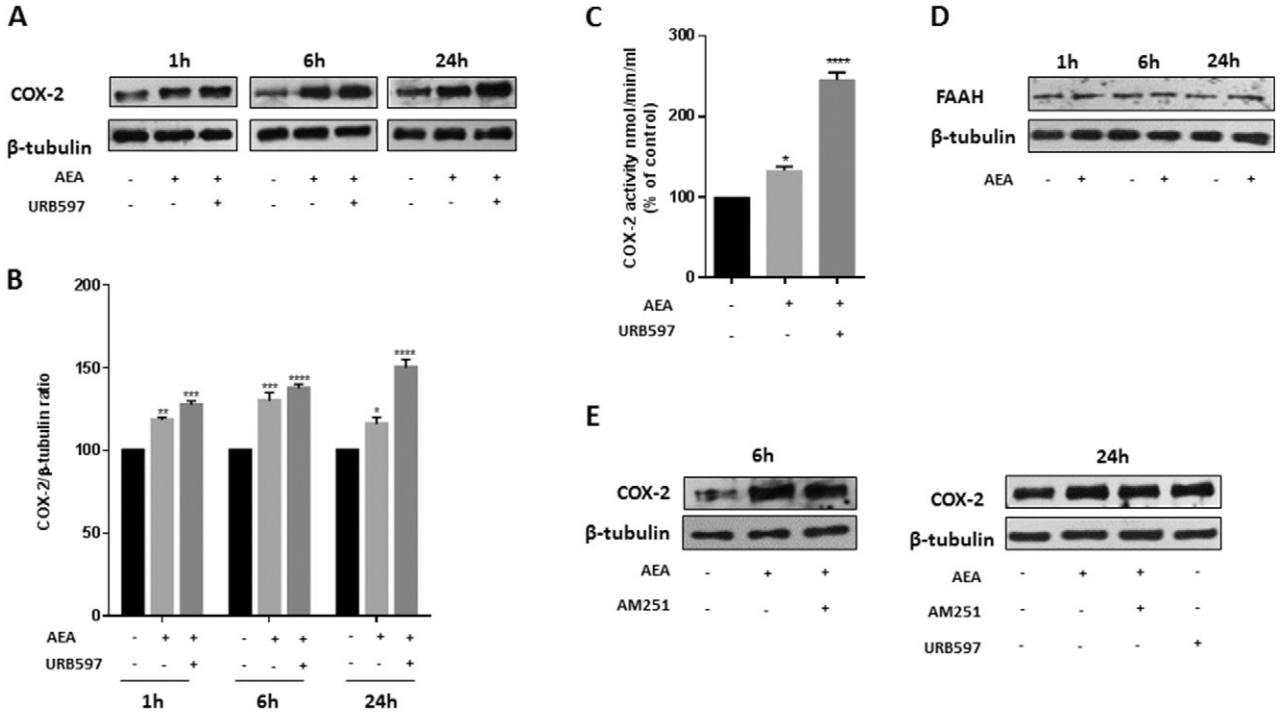
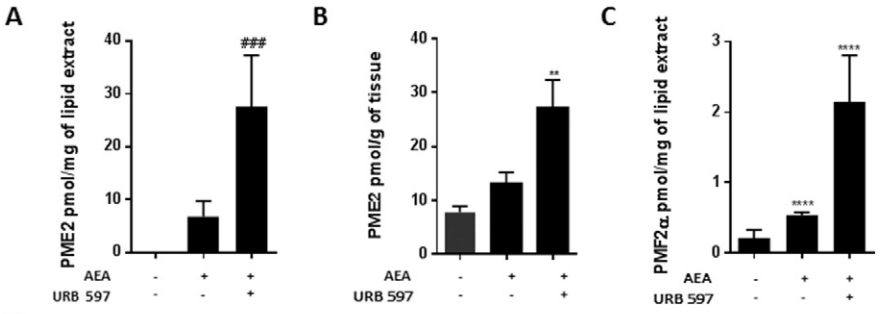


Fig. 1. Effect of AEA in COX-2 and FAAH protein expression. A) Time-course analysis of COX-2 expression after AEA treatment, and with or without FAAH inhibitor (URB 597), following 1 h, 6 h and 24 h of treatment by Western blot. B) Relative ratios of COX-2 and β-tubulin expression (used as a loading control). C) Analysis of COX-2 activity after 24 h of treatment. D) FAAH expression levels in the control group and in the presence of AEA, following 1 h, 6 h and 24 h of treatment. E) COX-2 expression upon treatment with AEA plus AM251 (CB1 antagonist) after 6 h and 24 h. Effect of URB 597 in COX-2 expression. (****P < 0.001, ***P < 0.01, **P < 0.1, *P < 0.05 vs. Control).



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	Relative Levels (ratio to control)			
	AEA	2-AG	PEA	OEA
AEA	70.51	1.75	1.26	1.55
AEA + URB 587	104.79	2.23	1.29	2.12

Fig. 2. Prostamides and endocannabinoid levels measured by LC–MS-IT-TOF. A) Levels of prostamide E2 (PME2) in rat decidual cells, after 24 h of treatment. B) PME2 levels in rat mesometrial decidual explants. Endogenous PME2 levels were detected in rat uterine explants. C) Levels of PMF2α, with or without URB 597, after 24 h of treatment in decidual cell cultures. D) Relative levels of endocannabinoids and N-acylethanolamines in decidual cells. Results are expressed as the ratio between the AEA or AEA co-incubated with URB 597 levels vs. Control (in the absence of AEA). (****P < 0.0001, **P < 0.01 vs. Control and ###P < 0.001 vs. AEA).

2.10. Statistical analysis

All assays were performed at least three times in triplicates. Statistical analysis was performed by using one-way ANOVA, followed by Tukey's ad-hoc post-test (GraphPad PRISM version 4.0, GraphPad Software, Inc., San Diego, CA, USA). All numerical data are expressed as mean ± SEM and differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Time-dependent AEA-induced increase in COX-2 expression and activity

In this study we performed a time-course analysis of COX-2 protein levels and activity, following AEA (10 μM) treatment (Fig. 1). Anandamide exposure led to an increase in COX-2 expression after 1 h of treatment, reaching peak levels after 6 h of incubation (Fig. 1A, B). This effect was slightly decreased after 24 h of treatment, though the enzyme activity remained significantly elevated by about 40% (Fig. 1C). Pre-incubation of AEA with a FAAH inhibitor (URB 597), for up to 24 h, led to a sustained and pronounced up-regulation of COX-2 protein levels and activity (Fig. 1B, C), suggesting that AEA and not its hydrolysis products was responsible for the effect. On the other hand, it is widely accepted that endogenous cannabinoids are primarily hydrolysed by fatty acid amide hydrolase (FAAH). Thus, we examined FAAH protein levels under the same experimental conditions (with or without AEA-treatment). Although FAAH is constitutively expressed in rat decidual cells, its expression was not affected by AEA-treatment (Fig. 1D). To further explore the role cannabinoid receptor 1 (CB1) on AEA-induced COX-2 expression, AEA was pre-incubated with AM 251 (a selective CB1 antagonist). As shown in Fig. 1E, the AEA-induced up-regulation of COX-2 was not reversed by CB1 antagonist (Fig. 1E). The incubation with URB 597 alone did not induce any alteration in protein expression (Fig. 1E).

3.2. Prostamide levels and eCB Levels

COX-2 directly oxygenates AEA into prostamides. As the involvement of COX-2 became apparent, we analysed prostamide levels by LC–MS-IT-TOF. In the control group of rat decidual cells, we could not detect PME2 (Fig. 2A). After 24 h treatment, with 10 μM of AEA, PME2 levels increased up to 7 pmol/mg. Moreover, PME2 levels were drastically increased (26 pmol/mg) when AEA was pre-incubated with URB 597 (Fig. 2A). To prove the relevance of AEA-metabolites in our experimental conditions, rat decidual explants were used to identify PME2 endogenously. In the control group, 8.05 pmol/g of PME2 were found, whereas AEA-treated explants presented in a 2-fold increase in PME2 levels, and pre-incubation with URB 597 led to a 4-fold increase compared to control (Fig. 2B). PMF2α was found in the control group of decidual cells. AEA-treatment induced an increase in PMF2α levels, and FAAH inhibition resulted in a 2-fold increase compared to control (Fig. 2C). As expected, in AEA plus URB 597-treated cells, AEA levels were significantly elevated, while the levels of other measured eCBs or eCB-related mediators were not affected (Fig. 2D).

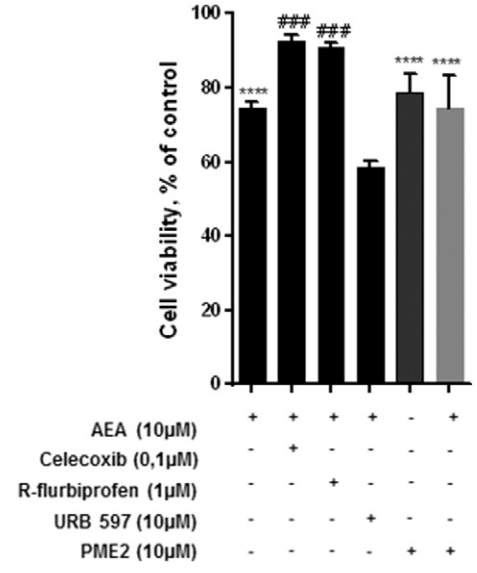

Fig. 3. Influence of COX-2 inhibition on AEA-induced cell death in rat primary decidual cell. Co-incubation of AEA with two selective COX-2 inhibitors, Celecoxib (0.1 uM) and R-Flurbiprofen (1 μM) significantly counteracted AEA-induced loss of cell viability. URB 598 plus AEA induced a higher decrease in decidual cell viability. (****P < 0.0001 vs. Control and ###P < 0.001 vs. AEA).

3.3. Influence of Celecoxib, R-Flurbiprofen and URB 597 on AEA-apoptosis

Next, the effect of COX-2 inhibition in AEA-induced loss of decidual cell viability was investigated. Cells were pre-incubated with two COX-2-selective inhibitors, Celecoxib (0.1 μM) and R-Flurbiprofen (1 μM), and viability of AEA-treated cells assessed by MTT assay. As shown in Fig. 3, both inhibitors significantly counteracted the AEA-induced decrease in cell viability. Moreover, COX-2 inhibition attenuated the features associated with the apoptotic process triggered by AEA, such as the drop in ΔΨm, the increase in ROS production and caspase-3/-7 and -9 activation (Fig. 4). In the presence of URB 597, AEA induced a more pronounced decrease in cell viability (of 40%) (Fig. 3).

3.4. Effect of major COX-2-derivatives on rat decidual cells

Additional experiments were performed to investigate the role of the major COX-2 derivatives on primary rat decidual cells, namely prostaglandin E2 and F2α (PGE2, PGF2α respectively) and prostamide E2 and F2α (PME2, PMF2α respectively). PGE2 (Fig. 5 A), PGF2α and PMF2α (data not shown) did not affect cell viability. On the contrary, the exposure of decidual cells to PME2 (0.01–50 μM) resulted in a loss of cell viability in a concentration-dependent manner (Fig. 5B). PME2 at 10 μM induced a decrease of 20% on cell viability. At this concentration, no LDH release was detected, and only concentrations higher than 25 μM caused the release of this enzyme (Fig. 5C). AEA co-administrated with PME2, did not have an addictive or synergistic effect on cell viability (Fig. 3). To further understand the process associated with the decrease on cell viability, nuclear morphology and mechanisms of apoptotic cell death were analysed. General morphology was preserved in PME2-treated cells. However, some cells presented chromatin condensation and the presence of apoptotic bodies, suggestive of the apoptotic process (Fig. 5D). As shown in Fig. 4, PME2 induced a 25% increase of the effector caspase-3/-7 activity. Moreover, PME2 engaged the intrinsic apoptotic pathway as assessed by an increase of 24% in caspase-9 activity and a drop in ΔΨm of 18%. PME2 led also to a significant increase of ROS production compared to control.

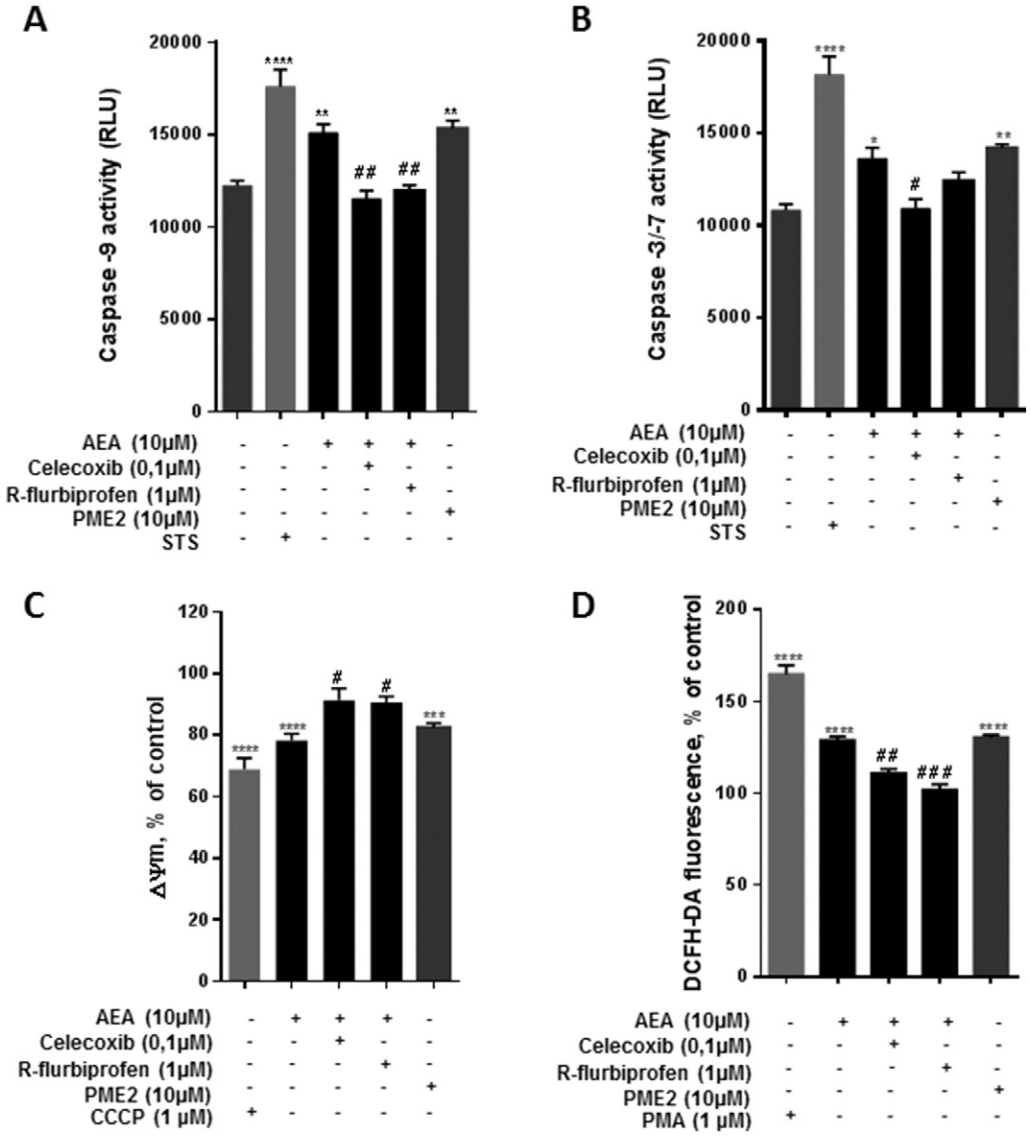

Fig. 4. Effect of PME2 and selective inhibition of COX-2 on decidual cell-induced apoptosis. PME2 triggers the intrinsic apoptotic pathway. Both inhibitors reverted the activation of the initiator caspase-9 (A) and effector caspase-3/-7 (B), the drop in mitochondrial membrane potential (ΔΨm) (C) and the increase in reactive oxygen species (ROS) production (D) caused by AEA. PME2 (10 μM) induced similar effects to AEA. (STS — staurosporine; CCCP — carbonyl cyanide m-chlorophenyl hydrazine; MPA — phorbol 12-myristate 13-acetate). (****P < 0.0001, ***P < 0.01, **P < 0.1, vs. Control and ###P < 0.001, ##P < 0.01, #P < 0.05 vs. AEA).

3.5. COX-2 induction by AEA occurs via MAPK signalling pathway and NF-kB

To understand the underlying mechanisms of AEA induced-COX-2 expression, we focused on the downstream signalling pathways, such as the MAPK cascade and NF-kB activation. As shown in Fig. 6, AEA produced an early and persistent phosphorylation of p38 kinase, with total p38 expression remaining unaltered. Following 24 h, when AEA was pre-incubated with URB 597, p38 phosphorylation returned to baseline (Fig. 6A, B). To reinforce the involvement of p38 MAPK as an upstream regulator of COX-2, decidual cells were pre-treated with SB202190, a specific p38 inhibitor, in the presence and absence of AEA. The inhibition of p38 activation attenuated the AEA-induced increase in COX-2 protein levels, after 6 h and 24 h treatment (Fig. 6C, D). As shown in Fig. 6, the activation of p42/44 was noted upon 6 h of AEA-treatment, but was independent of FAAH inhibition. Interestingly, at 24 h, p-p42/44 levels were similar to control cells, and a further drop was induced when AEA was co-incubated with URB 597. However, COX-2 up-regulation was not abolished by pre-incubation with PD98059, a specific p42/44 inhibitor (Fig. 6E). AEA-treated cells, both in the absence and presence of FAAH inhibitor, did not induce any alteration of JNK phosphorylation (Fig. 6A, B). As activation of NF-kB occurs via degradation of IkB-α, we examined IkB-α levels by Western blot. IkB-α protein levels were reduced by AEA-treatment (Fig. 7A). Furthermore, the immunocytochemistry analysis showed NF-kB translocation from the cytoplasm to the nucleus in AEA-treated cells, compared to control cells, suggesting the activation of NF-kB pathway (Fig. 7B).

4. Discussion

Uterine tissue remodelling is a highly coordinated process involving proliferation, differentiation and programmed cell death of endometrial cells. The regulation of apoptosis plays an important role in tissue homeostasis, as well as in endometrial remodelling. Recent research in the endocannabinoid system has unravelled eCBs as mediators in early pregnancy events, such as implantation [18], decidualization [19,20] and feto-placental development [21,22]. Previously, we have reported that the eCB machinery operates in the uterine environment and that AEA-induced apoptosis of decidual cells occurs through CB1 and via the activation of p38 pathway [15,19].

FAAH is the main hydrolytic enzyme, which degrades AEA into ethanolamine and AA. The latter is a substrate to cyclooxygenases (COX-1 and -2), to produce PGs. Alternatively, AEA is subjected to a direct oxidative metabolism by COX-2, but not COX-1, into a novel class of lipid mediators, named prostamides [6]. Amongst eCBs, AEA has been reported to regulate PG production in several systems, particularly in reproductive tissues [23]. For instance, it was described that AEA exerts opposite effects on PGE2 and PGF2α levels in uterine explants [24]. In addition, COX-2 mediates eCB-induced effects, particularly in apoptosis [25,26]. In mice, target disruption of COX-2, resulted in multiple reproductive impairments, including decidualization [14]. Moreover, a local increase of COX-2 expression was described during the decidual regression period [20,27]. Strikingly, in rats, COX-2 is mostly restricted to implantation sites, surrounding the implanting blastocyst, which may be associated with PGs production, a crucial factor for decidual cell reaction. However, COX-2 is hardly detected at the inter-implantation sites. Although the mechanisms underlying the COX-2-temporal and spatial pattern of expression remain unclear, it points toward COX-2 importance on early pregnancy events.

The present study unravels the role of COX-2 metabolic pathway in AEA-induced apoptosis in rat decidual cells. Regardless being constitutively expressed in decidual tissue, AEA led to a prompt and time-dependent increase in COX-2 protein levels. When AEA was pre-incubated with a FAAH inhibitor, COX-2 was persistently elevated. This indicates that AEA main hydrolysis product, AA, was not responsible for either AEA-apoptosis or up-regulation of COX-2. On the contrary, FAAH expression was not affected by AEA-treatment, suggesting that in decidual environment excess levels of AEA preferentially induced COX-2 up-regulation, over FAAH. Reinforcing the importance of COX-2 in AEA-induced effects, COX-2 inhibition, reverted the AEA-induced loss

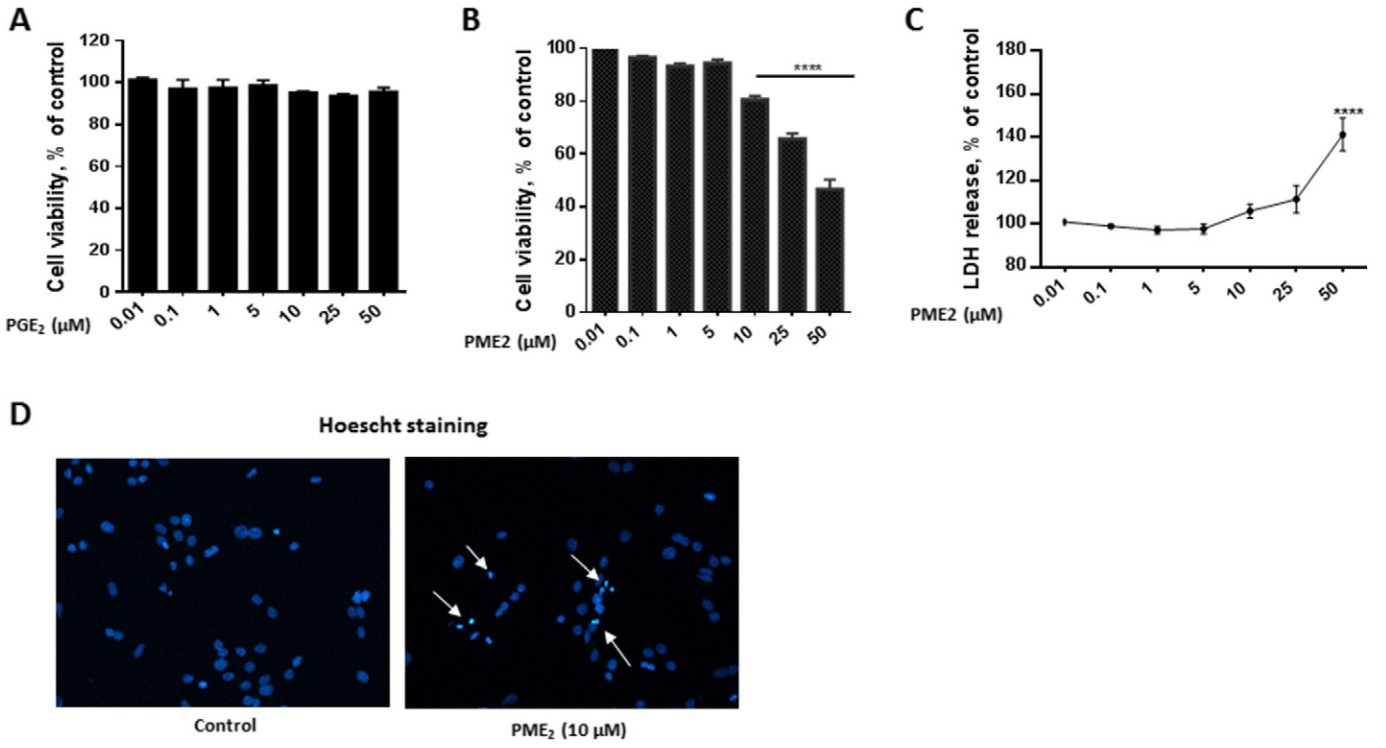

Fig. 5. Effect of prostaglandins and prostamides in cell viability. A) Prostaglandin E2 had no effect on decidual cell viability. B) PME2 (0.1–50 μM) induced a decrease in cell viability as assessed by the MTT assay. C) LDH enzyme release was observed for the highest PME2 doses (25–50 μM). D) PME2-treated cells presenting chromatin condensation by Hoescht staining (white arrows). (****P < 0.001 vs. Control).

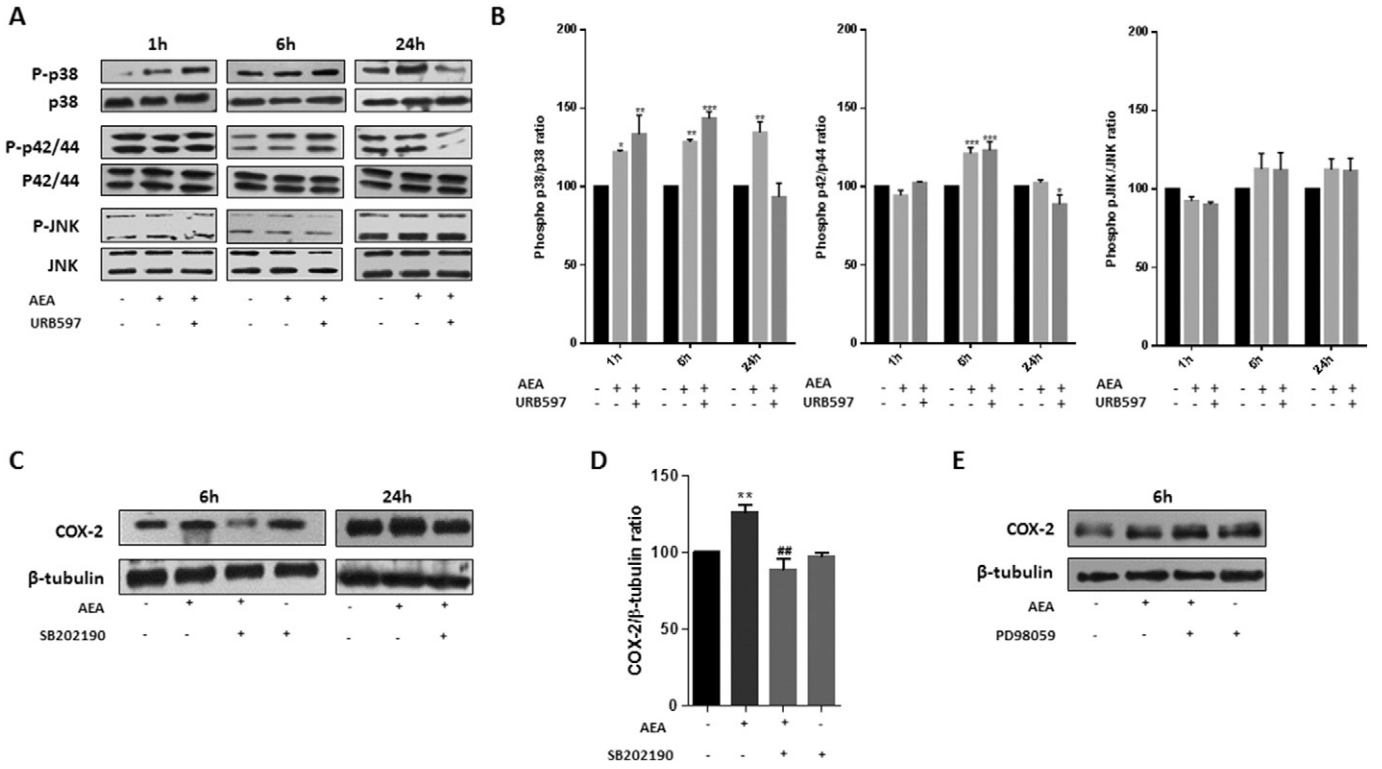

Fig. 6. Signalling pathways involved in AEA-induced COX-2 expression. A) Time-course expression of p38 MAPK, p44/42 and JNK signalling molecules after treatment with AEA, or AEA pre-incubated with URB 597, by Western blot. B) Relative ratios of phospho-p38 MAPK, phospho-p44/42 and phospho-JNK to the respective non-phosphorylated form. C) AEA pre-treated with SB202190, a selective p38 inhibitor, reduced COX-2 expression, after 6 h and 24 h of treatment and D) relative ratios of COX-2 and β-tubulin. E) AEA pre-treated with PD98059, a p42/44 inhibitor, did not affect COX-2 expression after 6 h of treatment. In the absence of AEA, COX-2 expression was not affected. β-Tubulin was used as a loading control. (***P < 0.001, **P < 0.01, *P < 0.05 vs. Control and ##P < 0.01 vs. AEA).

of cell viability, and counteracted the major features of AEA-apoptotic cell death in decidual cells. In particular, R-Flurbiprofen, which preferentially inhibits the COX-2-mediated oxidation of AEA vs. AA [28,29], reverted apoptosis triggered by AEA.

The strongest piece of evidence in favour of the physiological relevance of the COX-2 oxidative metabolism of AEA on decidual cells was the identification of prostamides as endogenous lipid mediators in decidual tissue, particularly PME2 and PMF2α. We also found that

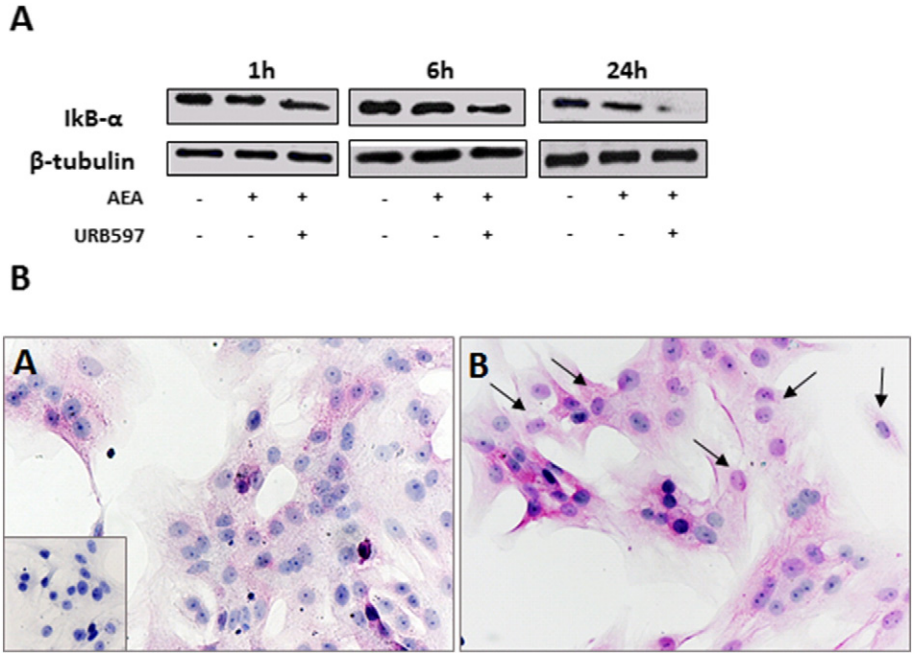

Fig. 7. Activation of NF-kB pathway by Western blot and immunocytochemistry analysis. A) Decreased IkB-α levels were found in the presence of AEA and in the presence of URB 597. β-Tubulin was used as a loading control. B) Immunolocalization of NF-kB in decidual cells in the control group (A) and AEA-treatment (B) for 24 h of treatment. In AEA-treated cells note the nuclear expression of NF-kB (black arrows). Negative control obtained by omitting the primary antibody. (Original magnification, ×400).

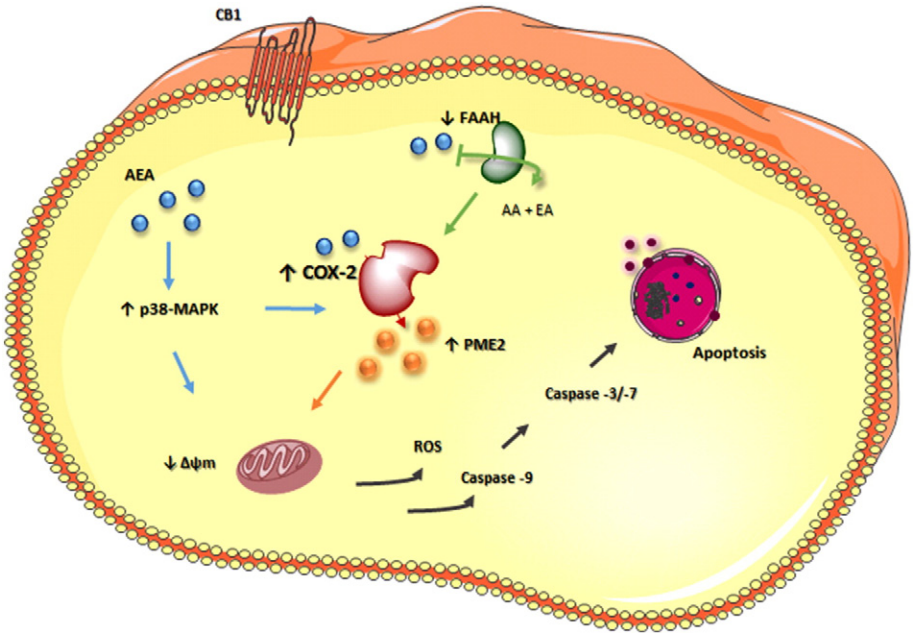

Fig. 8. AEA-induced decidual cell apoptosis involves COX-2-oxidative metabolism. Proposed mechanism of action. Anandamide (blue circles), in a mechanism independent of CB1, but through p38 MAPK activation, induces an up-regulation of COX-2 expression and local formation of prostamides, resulting in decidual cell apoptosis. Moreover, when FAAH activity is deregulated (in this case mimicked by FAAH inhibition), altered AEA levels potentiate COX-2 oxidative metabolism and a further increase in PME2 (orange circles) and cell apoptosis. PME2-induced apoptosis on decidual cells, in a mechanism similar to AEA. (Arachidonic acid (AA); anandamide (AEA); cannabinoid receptor 1 (CB1); cyclooxygenase-2 (COX-2); ethanolamine (EA); mitochondrial membrane potential ($\Delta\psi m$), prostamide E2 (PME2); reactive oxygen species (ROS)).

decidual cells produced significant amounts of PME2 in response to AEA, particularly when FAAH was inhibited. Although the formation of COX-2-derived metabolites of eCBs has been known, their biological effects remain to be fully elucidated. To date, they have been reported as mediators in inflammation [16,30], pain [16], apoptosis [25,26] and adipocyte differentiation [29]. In our model, the pro-apoptotic action of AEA was mimicked by the major COX-2 derivative, i.e. PME2, suggesting that it may account for AEA-induced impairment on decidual stability. On the other hand, when added together, PME2 and AEA, did not increase the AEA-induced loss of cell viability, suggesting a possible common molecular mechanism.

In addition, we disclosed the molecular mechanism underlying AEA-induced COX-2 up-regulation. Previously, we reported that AEA-induced apoptosis in decidual cell was mediated through p38 activation [19] and that CB1 antagonist partially attenuated this effect [15]. Presently, we further demonstrate that p38 MAPK activation also mediates the AEA-induced effect on COX-2 protein levels. Interestingly, while short term exposure to FAAH inhibition induced p-p38 MAPK, long term exposure prevented p38 activation [31,32]. Although different pathways may be involved, this phenomenon was previously reported and may suggest a negative feedback loop to dephosphorylate activated p38 [31], or as the level of total p38 was constant, the observed changes might reflect a drop of kinase activity [32]. On the other hand, although AEA induced an early activation of p42/44, the inhibition of this pathway did not prevent the effect upon COX-2 regulation. Phosphorylation of p42/44 is generally associated with cell survival, proliferation and differentiation, and its inhibition may result in apoptosis [32]. The reduction of IkB-α levels, as well as the translocation of NF-kB to the nucleus after AEA treatment, also indicates the involvement of NF-kB pathway.

Although COX-2 is not considered a major eCB-metabolic enzyme, it may compete with FAAH for eCB as substrates, and utmost our results shed light on the COX-2's physiological influence on AEA-signalling. Besides the drastic and sustained up-regulation of COX-2, FAAH inhibition led to a significant decrease of decidual cell viability and a considerable increase of PME2 levels. In $FAAH^{-/-}$ female mice, preimplantation, embryo development and pregnancy outcome are adversely affected [33]. In a similar context, low FAAH activity and high AEA plasma levels were apparent in recurrent miscarriage or poor implantation in women undergoing in-vitro fertilization [5]. Also, low or absent FAAH expression was associated with spontaneous miscarriage [34]. Thus, on the basis of ours findings, it may be suggested that increased levels of AEA, due to aberrant biosynthesis and/or deregulation of FAAH activity (in this case, mimicked by FAAH inhibition), might potentiate endogenous prostamide signalling.

It has been postulated that eCBs are involved in a network of hormones, cytokines and other lipids, such as prostaglandins, which modulate cellular events critical for early pregnancy development. Albeit AEA levels in the uterus are higher than those in any other organ, low levels are required in the receptive uterus or at implantation sites [35]. Maternal eCB tissue levels are strictly regulated to allow implantation and establishment of pregnancy. Given the COX-2 differential pattern of expression during decidual remodelling process, our results underlie how the endometrium may represent a niche in which COX-2 acts as a central gatekeeper toward AEA decidual signalling and in situ levels.

In overall, our study unveils that in the decidual environment, altered levels of AEA induce COX-2 upregulation, through p38 MAPK, resulting in the local increase of prostamides and decidual apoptosis (Fig. 8). Moreover, it is tempting to speculate that when FAAH activity is disrupted, excess AEA levels may interfere with COX-2 regulation, and impair decidual stability. As decidual cells may be central controllers of uterine endocannabinoid levels during early pregnancy, AEA metabolic pathway must be tightly regulated during decidual remodelling process. Otherwise, a disruption on AEA signalling might be decisive for decidual cell fate and result in deleterious effects during pregnancy or in disorders like miscarriage and infertility.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgments

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Manuscript III:

The endocannabinoid anandamide impairs human decidualization

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The endocannabinoid anandamide impairs human decidualization

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Abstract

Endocannabinoids (eCBs) are endogenous lipid mediators that bind and activate the cannabinoid receptors (CB1, CB2). These compounds along with their receptors, a membrane transporter and the respective metabolic enzymes form the endocannabinoid system (ECS). Several eCBs have been discovered with emphasis on anandamide (AEA). They are involved in several basic biological processes like energy balance, immune response and reproduction.

A well-coordinated process, named decidualization, occurs during the secretory phase of the human menstrual cycle, which involves intense proliferation and differentiation of endometrial stromal cells into decidual cells is a prerequisite for the establishment and progression of pregnancy. In rat, we have previously reported that AEA impairs the progression of the decidual process, although its role on human decidualization is yet unknown. In this study, it was employed a telomerase-immortalized human endometrial stromal cell line (St-T1b) and primary cultures of human decidual fibroblasts (HdF) from term placenta, in which the ECS was characterized in both non-differentiated and differentiating cells, by immunoblotting and qRT-PCR experiments. It was shown that St-T1b cells express CB1 receptor, but not CB2, though both cannabinoid receptors are expressed in HdF cells. In addition, it was detected an increase in the expression of fatty acid amide hydrolase (FAAH), the main degrading enzyme of AEA, during decidualization. Furthermore, the impact of deregulated levels of AEA was explored during the decidualization process. Two major outcomes are highlighted in this study. Anandamide inhibits cell proliferation, interferes with cell cycle progression and induces polyploidy. Moreover, through CB1 binding receptor, AEA also impairs cell differentiation. Therefore, AEA is proposed as a novel modulator of human decidualization. Our findings may provide wider implications, in a clinical perspective, as deregulated levels of AEA, due to Cannabis sativa consumption or altered expression of the metabolic enzymes, may negatively regulate human endometrial stromal cell proliferation and decidualization with an impact on human reproduction and (in)fertility.

Keywords: endocannabinoids, anandamide, decidualization, proliferation, differentiation

Introduction

The highly dynamic nature of human endometrium is a hallmark of reproductive biology. Human endometrial stromal cells (HESCs) undergo waves of proliferation and differentiation into morphologically and functionally distinct cells called decidual cells. This process, named decidualization, occurs in women during the secretory phase of the menstrual cycle and prepares a receptive uterus for implantation. The decidual process is triggered by sustained activation of the second messenger cyclic adenosine monophosphate (cAMP) and the protein kinase A pathway (PKA), which in turn sensitizes stromal cells to progesterone [1]. In addition to uterine angiogenesis and homeostasis [2], decidual cells are responsible for embryo recognition [3], regulation of trophoblast invasion and modulation of immune and oxidative stress response at feto-maternal interface [4]. Impaired decidualization, as a result of poor hormone responsiveness and/or impaired endometrial stromal cell growth and/or differentiation, is often associated with implantation failure, unexplained infertility, recurrent spontaneous miscarriage, and pregnancy disorders, such as preeclampsia or intrauterine growth retardation and premature birth [5]. The endocannabinoids (eCBs), and particularly anandamide (AEA), the best studied eCB, are emergent modulators of several pathophysiological processes. Besides the endogenous cannabinoids, the endocannabinoid system (ECS) comprises the cannabinoid receptors (CB1 and CB2), the enzymes involved in the biosynthesis and degradation of eCBs, and a purported transporter [6]. AEA is mainly synthesized by N-arachidonoylphosphatidylethanolamine hydrolyzing phospholipase D (NAPE-PLD), and, subsequently, hydrolyzed to arachidonic acid and ethanolamine by the enzyme fatty acid amide hydrolase (FAAH) [6]. The latter is considered the

major metabolic gatekeeper for AEA uterine levels throughout menstrual cycle and pregnancy [7, 8]. AEA modulates basic biological processes, including the choice between cell proliferation, death and differentiation [9]. In the rat model, it has been demonstrated that AEA interferes with decidual cell turnover by inducing apoptosis [6] and limits uterine stromal cell differentiation [10]. It has also been shown that AEA induces apoptosis in cytotrophoblast cells [11]. Nevertheless, the underlying mechanisms that govern the process of human endometrial decidualization and the involvement of the eCBs in this process remain poorly understood. Therefore, in this work we aimed to investigate the role of the main eCB, AEA on *in vitro* decidualization process in two cell culture models, an immortalized human endometrial stromal cell line (St-T1B) and human decidual fibroblasts (HdF) derived from human term placenta. The ECS was characterized in non-differentiated and in cells undergoing decidualization, and the role of AEA in these cells was investigated.

Material and methods

St-T1b cell line culture

The human endometrial stromal cell line (St-T1b) was kindly supplied by Dr. Birgit Gellersen from Endokrinologikum Hamburg, Hamburg, Germany. This cell line was obtained from uterine biopsy samples at the time of hysterectomy from benign gynaecological disorders and immortalized with human telomerase (hTERT) [12]. Cells were maintained at 37 °C with 5% CO_2 in DMEM/F-12 medium (Gibco Invitrogen Corporation, CA, USA) supplemented with 10% charcoal-stripped fetal bovine serum (CT-FBS) (Gibco Invitrogen Corporation, CA, USA), 1 nM 17-β- estradiol (E_2) (Sigma-Aldrich Corporation, MO, USA), 1 µg/ml Insulin (Sigma-Aldrich Corporation, MO, USA) and 2% penicillin-streptomycin-amphotericin B (Gibco Invitrogen Corporation, CA, USA). Initially, cells were incubated for 24 h in complete medium and then experiments were performed in DMEM/F-12 with 2 % CT-FBS.

Primary cultures of human decidual fibroblast (HdF)

The protocol for the isolation of cells from term decidua was based on Richards et al, 1995 [13]. Caesarean or vaginal delivery normal human placentas (38–40 weeks of gestation) from Caucasian women, living in Porto region and aged 24–36 years old, were obtained from Centro Materno-Infantil do Norte, Porto. All the procedures using term placentas were performed according to the Ethical committee of Centro Materno-Infantil do Norte. The decidua basalis was scraped from the chorionic membrane, dissected into small pieces and enzymatically digested in PBS with collagenase (1 mg/ml) for 1 h at 37 ºC with gentle shaking. The suspension was then centrifuged at 260 *g* for 6 min. The pellet was ressuspended with ammonium chloride (0.84%) solution for red blood cells lysis at 37 ºC. The resulted suspension was filtered through a 40 µm nylon mesh and centrifuged at 260 *g* for 10 min. The isolated cells were maintained at 37 ºC with 5% CO_2 in DMEM/F-12 medium supplemented with 10% CT-FBS, 1 nM E_2, 1 µg/ml insulin and 2% penicillin-streptomycin-amphotericin B. After 24 h, the non-adherent cells were removed and the cells were grown to confluence and sub-cultured. The purity of primary HdF was confirmed by immunocytochemical analysis for the cytoskeletal proteins vimentin and cytokeratin-7, a fibroblast and an epithelial cell marker, respectively (Fig. 1).

Differentiation treatment

In vitro differentiation was induced in minimal medium MM1 (DMEM/F-12 medium supplemented with 2% CT-FBS and 2% penicillin-streptomycin-amphotericin B) containing 1 µM medroxyprogesterone acetate (MPA; Sigma-Aldrich, MO, USA), 10 nM of E2 and with 0.5 mM of 8-Bromoadenosine 3',5'-cyclic monophosphate (cAMP, Sigma-Aldrich, MO, USA) or 0.05 mM cAMP, for St-T1b and HdF, respectively. The differentiation process was induced for 3 or 6 days (d), and the medium was changed every 3 d. Cell differentiation was confirmed by morphological analysis and mRNA levels of prolactin (PRL) and the insulin-like growth factor binding protein-1 (IGFBP-1), the main secretory products of decidual cells, were evaluated by qRT-PCR. In order to investigate the role of AEA during the decidualization process, cells were treated with the differentiation medium (MM1 + cAMP, E_2 and MPA) in the presence or absence of AEA (10 µM) (Tocris Bioscience, MN, USA). To explore the role of CB receptors, cells were pre-incubated with the CB1 antagonist (AM281) and the CB2 antagonist (AM630) for 30 min before the addition of AEA (10 µM). At this concentration, the antagonists *per se* did not affect neither the decidual response nor cell viability. Non-differentiated cells treated with MM1 were used as a control. The results are the mean of at least three independent experiments carried out in triplicate.

Cell viability and cell proliferation

HdF and St-T1b cells were plated at a density of 2.5×10^3 cells/well in 96-well plates and treated with AEA (10 µM) in non-differentiated medium (minimal medium, MM1: DMEM/F-12 medium

supplemented with 2% CT-FBS and 2% penicillin-streptomycin-amphotericin B), and with differentiation medium (MM1 + cAMP, E_2 and MPA). Cell viability was assessed by MTT assay after 6 d of treatment. The yellow tetrazole MTT (0.5 mg/ml final concentration) (Sigma-Aldrich, MO, USA) was added and cells were incubated at 37 ºC for 3 h. The resultant purple formazan was dissolved in a solution of dimethylsulfoxide:isopropanol (3:1) and spectrophotometrically quantified at 540 nm. To study the effects of AEA on DNA synthesis, ^{3}H-thymidine incorporation assay was performed. In the last 32 hours of each exposure time, ^{3}H-thymidine (0.5 µCi) (Amersham, BU, UK) was added to cells. Cells were harvested and, after addition of a scintillation cocktail, ^{3}H-thymidine incorporation was determined in a scintillation counter (LS 6500, Beckman Instruments). Results are expressed as relative percentage of the untreated control cells. The results are the mean of at least three independent experiments carried out in triplicate.

Morphological studies

Morphological alterations induced upon differentiation treatment were evaluated by Giemsa staining. Both cell types were plated at a density of 1.5 x 10^4 cells/well in 24-well culture plate, and cells were exposed to differentiation treatment for 6 d. After treatment, cells were fixed with 4% p-formaldehyde in PBS for 20 min at 4 ºC, and stained with Giemsa for 30 min. Cells were observed under the microscope Eclipse E400, Nikon equipped with image analysis software LeicaQwin (Cambridge, UK).

qRT-PCR analysis

For qRT-PCR analysis, cells were seeded in 6-well plate, at a cellular density of 5 x 10^4 cells. After treatment, the cells were collected in TRIzol® reagent (Gibco/Invitrogen Corporation, Carlsbad, CA, USA) and RNA and protein were extracted according to the manufacturer's instructions. St-T1b and HdF cells were treated with the differentiation medium for 3 and 6 d. To study the role of the AEA and the involvement of CB receptors on the decidualization process, cells were treated with differentiation medium in the absence or presence of AEA (10 µM) plus CB1/CB2 antagonists for 6 d. The assessment of gene transcription was carried out by q-RTPCR. RNA was quantified in the NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE, USA), its quality was evaluated with the Experion RNA StdSens Kit (Bio-Rad Laboratories, PA, USA) and was analysed with Experion analytical software (Bio-Rad Laboratories, PA, USA). cDNA was obtained by reverse transcription of RNA using the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, USA) and it was subsequently amplified with specific primers, using KAPA SYBR® FAST qPCR Master Mix 2x Kit (Kapa Biosystems, Woburn, MA, USA) in MiniOpticon Real-Time PCR Detection System (Bio-Rad Laboratories, PA, USA), according to the kit protocol. Table 1 resumes the primer sequences and the qRT-PCR conditions. The specificity of PCR product amplification was assessed by analysis of the melting curve. Gene expression was normalized with two housekeeping genes, GAPDH and β-2 microglobulin, and their analysis was achieved by the calculation of ΔΔCT values. The results are the mean of at least four independent experiments carried out in triplicate.

Immunocytochemistry

For immunocytochemistry studies, cells were seeded in 24-well plate, at a cellular density of 1.5 x 10^4 cells, and fixed with cold methanol after treatment with differentiation stimulus for 6 d. The expression of proteins was analysed using an avidin–biotin alkaline phosphatase complex technique (Vectastain ABC kit, Vector Laboratories, CA, USA). The non-specific binding sites were blocked and slides were incubated with primary antibodies vimentin (1:500; sc 6260, St Cruz Biotechnology, CA, USA) and cytokeratin-7 (1:100; M 7018, Dako, GP, Denmark) overnight at 4 ºC. It was followed by incubation with biotinylated secondary antibody and with Vectastain ABC-AP reagent, according to the manufacturer's instructions. The reaction was developed with Sigma Fast RedTM tablets (Sigma-Aldrich, MO, USA). For the counterstaining it was used Mayer's haematoxylin solution (Sigma-Aldrich, MO, USA) and slides were mounted in Aquamount medium (BDH Laboratory Supplies, Pl, UK). Negative controls were performed by the replacement of the primary antibodies by rabbit IgG.

Cell cycle analysis

Cells were plated at a density of 5 x 10^4 cells in 6-well dishes and exposed to the differentiation medium in the absence or presence of AEA (10 µM), plus CB1/CB2 antagonists for 6 d. After tripsinization, cells were centrifuged (260 *g*, 5 min, 4ºC), resuspended in PBS and fixed in 70% cold ethanol. After fixation, cells were centrifuged and resuspended in 0.5 ml of DNA staining solution (5 µg/ml Propidium Iodide (PI), 0.1% Triton X-100 and 200 µg/ml Dnase-free Rnase A) overnight at 4 ºC. DNA content was analysed by flow cytometry based on the acquisition of 40 000 events (with a

threshold of 100 000) in a BD Accuri™ C6 (Becton–Dickinson Jose, CA, U.S.A) equipped with BD Accuri C6 software. Detectors for the three fluorescence channels (FL-1, FL-2 and FL-3) and for forward (FSC) and side (SSC) light scatter were set on a linear scale. Debris and cell aggregates were gated out, and singlets and doublets cells were analysed using a two-parameter plot of FL-2-Area to FL-2-Width of PI fluorescence. Data was analysed using BD Accuri C6 Software. The anti-proliferative effects were indicated by the percentage of cells in G_0/G_1, S and G_2/M phases of cell cycle. The results are the mean of at least three independent experiments carried out in triplicate.

Western blot

St-T1b cells and HdF (both non-differentiated and undergoing differentiation) were treated in the presence or absence of AEA (10 μM). Cell extracts were prepared in a potassium phosphate buffer containing a cocktail of protease inhibitors. Protein concentrations were measured by Bradford assay. Samples (20 μg) were run on 10% SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were incubated with primary antibody (Table 2) at 4 °C overnight. Membranes were then washed and incubated with goat anti-rabbit IgG horseradish peroxidase–conjugated antibody, and detected by enhanced chemiluminescence Western Bright™ ECL and exposed to x-ray film. β-tubulin was used as a loading control.

Evaluation of mitochondrial transmembrane potential (Δψm)

For mitochondrial transmembrane potential (ΔΨm) assessment, non-differentiated and cells undergoing decidualization were treated with or without AEA (10 μM) and stained with $DiOC_6(3)$ (30 nM) for 30 min. PI (5 μg/ml) was added prior to analysis to discriminate among live cells that stain only with $DiOC_6(3)$ ($DiOC_6(3)^+/PI^-$), early apoptotic cells that lost the ability to accumulate $DiOC_6(3)$ ($DiOC_6(3)^-/PI^-$), and late apoptotic/necrotic cells that stain only with PI ($DiOC_6(3)^-/PI^+$). As positive control, cells were incubated with 15 μM of the mitochondrial depolarizant agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich, MO, USA). The ΔΨm was analysed by flow cytometry based on the acquisition of 20 000 events (with a threshold of 100 000) in a BD Accuri™ C6 (Becton–Dickinson Jose, CA, U.S.A) equipped with BD Accuri C6 software. Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale, while the three fluorescence channels (FL-1, FL-2 and FL-3) were set on logarithmic scale. FL-1 was used to measure DiOC6(3) at green fluorescence and FL-2 and FL-3 to measure PI red fluorescence. Data were analysed using BD Accuri C6 Software. The results are the mean of three independent experiments carried out in triplicate.

Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey test for multiple comparisons (GraphPad PRISM version 4.0, GraphPad Software, Inc., CA, USA). All numerical data are expressed as mean ± SEM and differences were considered to be statistically significant when $p < 0.05$.

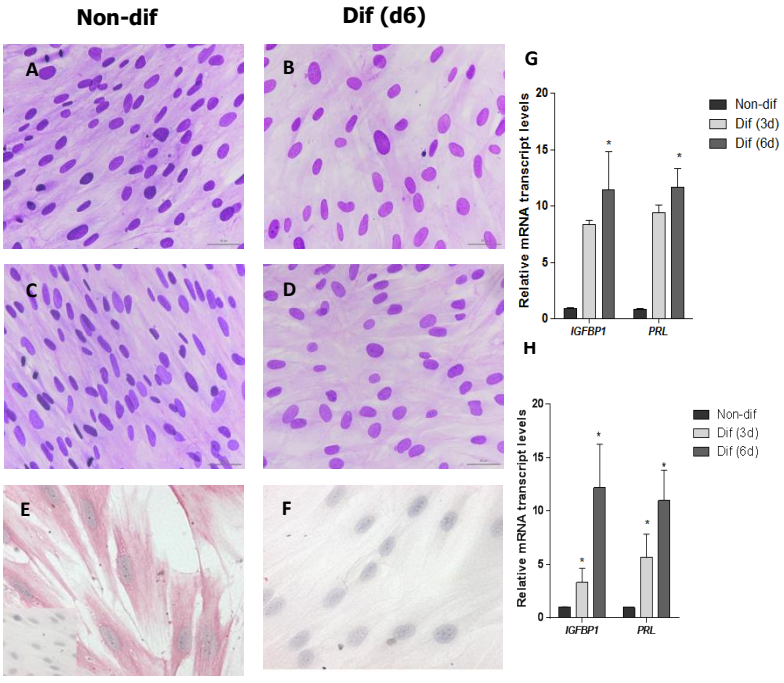


Figure 1. Study of the decidualization process in St-T1b and HdF cells after 3 and 6 days of exposure to decidualization stimulus. Morphological analysis in St-T1b cell line, by Giemsa staining, in non-differentiated (non-dif) (A) and in cells exposed for 6 d with differentiation stimulus (dif) (B). Similar to St-T1b cells, in HdF, non-differentiated cells (C) maintain the fibroblastic spindle-shape, whereas after 6 d of exposure to decidual treatment, differentiated cells (D) became larger, rounded nucleus and with increased amount of cytoplasm. The purity of primary HdF was confirmed by the expression of the cytoskeletal proteins vimentin (E) and cytokeratin (F) by immunocytochemistry, respectively, a fibroblast and an epithelial cell marker. The mRNA levels of *IGFBP-1* and *PRL* were evaluated by qRT-PCR. It was observed a significant increase in both St-T1b (G) and HdF cells (H) cells, particularly after 6 days of decidualization stimulus. (*$p < 0.05$ vs. Control).

Results

Expression of decidual markers in decidualizing St-T1b and HdF cells

In order to study the differentiation process, two endometrial cell culture systems were employed, a telomerase-immortalized human endometrial stromal cell line (St-T1b) and a primary culture of human decidual fibroblasts (HdF). In both St-T1b and HdF cell models, differentiation was induced for 3 and 6 days (d). The differentiation process was assessed by the mRNA levels of specific decidual markers, the prolactin (PRL) and the insulin like growth binding protein-1 (IGFBP-1) and by morphological analysis (Fig. 1). Giemsa staining showed that non-differentiated cells from St-T1b cell line (Fig. 1 A) and HdF cells (Fig. 1 C), maintained the elongated fibroblastic spindle-shape, whereas decidualizing cells became larger, with increased amount of cytoplasm and rounded nucleus in both St-T1b cell line (Fig. 1 B) and HdF cells (Fig. 1 D). As *PRL* and *IGFBP-1* are widely used biochemical markers of decidualization, gene transcription was analysed in non-differentiated and in differentiating cells upon 3 and 6 d of differentiation stimulus by qRT-PCR. In both St-T1b (Fig. 1 G) and HdF cells (Fig. 1 H) the mRNA levels of the decidual markers were significantly increased, particularly in cells undergoing decidualization for 6 d. Therefore, the subsequent studies were only proceeded in cells undergoing differentiation during 6 d.

Characterization of the Endocannabinoid System in St-T1b and HdF cells

The expression of major enzymes involved in AEA metabolism, NAPE-PLD and FAAH, and the cannabinoid receptors, CB1 and CB2, were investigated at mRNA and protein levels by qRT-PCR and Western Blot analysis, respectively (Fig. 2). In St-T1b cells (Fig. 2 A, B) mRNA and protein levels of CB1 receptor were not affected upon differentiation, and it was found that these cells do not express CB2 receptor. No differences were observed in NAPE-PLD expression, while FAAH expression was up-regulated at both gene transcription and protein levels in cells undergoing differentiation. In HdF cells, it was found a fully ECS biochemical machinery (Fig. 2 A, C). CB1 transcript and protein levels were up-regulated in differentiating cells, while CB2 expression remained unaffected. Regarding the metabolic enzymes, NAPE-PLD expression remained constant during *in vitro* differentiation, whilst FAAH was up-regulated at mRNA transcript and protein levels, similarly to St-T1b cell line.

Anandamide effect on human endometrial cell viability and cell proliferation

Non-differentiated and cells exposed to the differentiation medium were cultured for 6 d in the presence or absence of AEA (10 μM) and cell viability was assessed by MTT assay.

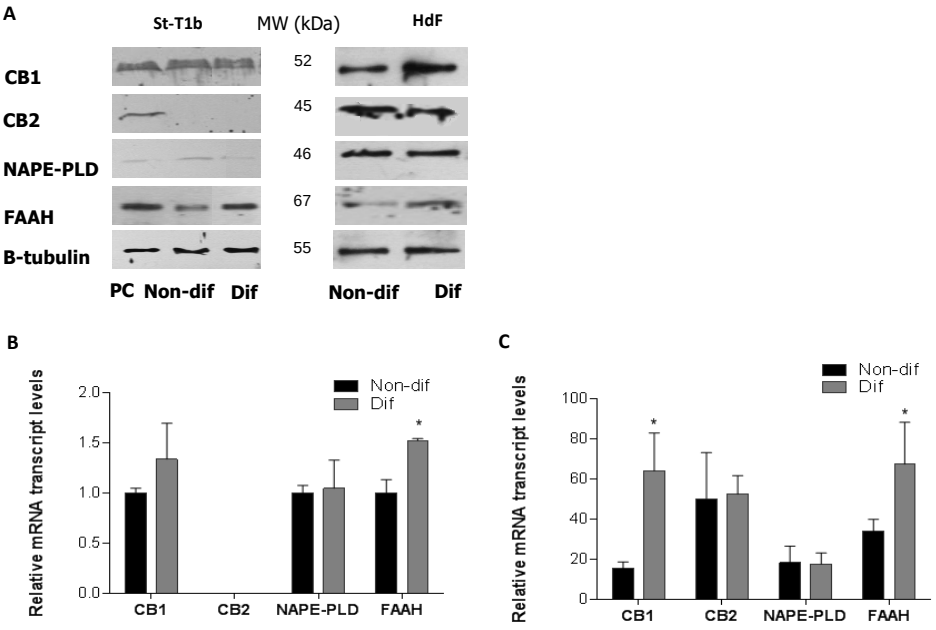


Figure 2. Characterization of the Endocannabinoid system (ECS) by Western blot and qRT-PCR in non-differentiated (non-dif) and differentiating cells (dif) treated for 6 days with differentiation stimulus. (A) Protein levels of the principal members of the ECS, in both St-T1b and HdF cells. In HdF, CB1 expression was increased in cells undergoing decidualization. St-T1b cell line, does not express CB2 receptor. mRNA transcript levels of the CB receptors and main metabolic enzymes of AEA, FAAH and NAPE-PLD in St-T1b cell line (B) and HdF cells (C). It was found that the gene encoding for FAAH was significantly up-regulated in decidualizing cells, in both cell models. (**p* < 0.05 vs. Control).

In the culture conditions used, AEA did not affect cell viability, neither on non-differentiated (Fig. 3 A, D) nor on cells undergoing differentiation (Fig. 3 B, E) in both St-T1b and HdF cell models. This concentration was chosen because it does not affect cell viability and it was previously reported that impairs stromal cell differentiation in the rat model. The decidualization process involves extensive proliferation and differentiation of HESCs, thus, to investigate the role of AEA on cell proliferation, ^{3}H-thymidine incorporation was examined in both non-differentiated and differentiating cells, in the presence or absence of AEA (10 μM) (Fig. 3 C, F for St-T1b and HdF cell models, respectively). In non-differentiated cells, AEA caused a decrease in ^{3}H-thymidine incorporation of 28% in St-T1b and of 30% in HdF cells, compared to non-treated cells. As expected, cell proliferation was reduced in decidualizing cells compared to non-differentiated cells. However, in cells undergoing differentiation, AEA-treatment markedly decreased the rate of DNA synthesis by 40% compared to differentiating cells, in both cell models.

In order to understand this anti-proliferative effect, it was further explored the AEA-impact on cell cycle progression (Fig. 4). In non-differentiated cells, AEA treatment led to a cell cycle arrest at G_2/M phase, both in St-T1b (12,37%) and in HdF cells (34,38%) compared to control (8,51% and 30,16%, respectively). Upon differentiation, stromal cells cease to proliferate, thus, the differentiation stimulus, caused a significant increase of cells arrested at the G_0/G_1 phase and a decrease in the cellular content at the S phase, in both cell models.

In St-T1b cells exposed to AEA during differentiation treatment, it was found a decrease in the cellular content at the G_0/G_1 phase, compared to decidualizing cells (84,77% vs. 92,15%, respectively) and a significant arrest at G_2/M phase of 7% compared to decidualizing cells (Fig. 4 A).

A similar pattern of cell cycle distribution was found in HdF cells (Fig. 4 D). Consistently, during differentiation, in HdF cells, AEA-induced a decrease of cellular content at the G_0/G_1 phase compared to differentiating cells (5,32% vs. 70,51%, respectively), and a significant cell cycle arrest at the G_2/M phase of 13,49%, when compared to cells undergoing differentiation (40,40 vs. 26,91%, respectively).

Analysis of decidualizing cells treated with AEA plus AM281, a selective CB1 antagonist, showed a cell cycle distribution similar to control differentiated cells. Moreover, when comparing to AEA-treated differentiating cells it was observed a retention at G_0/G_1 phase and a decrease on G_2/M cellular content, in both cell models. Unexpectedly, it was noticed by Giemsa staining that in AEA-treated cells there was an increased number of bi- or multi-nucleated in cells (Fig. 4 C, F). The flow cytometric analysis, further confirmed the increased number of multi-nucleated cells (> 2n) upon AEA-treatment (Fig. 4 A, D). In both non-differentiated and decidualizing cells, AEA-treatment induced at least a 2-fold increase on the number of bi-nucleated cells, in St-T1b and HdF cells.

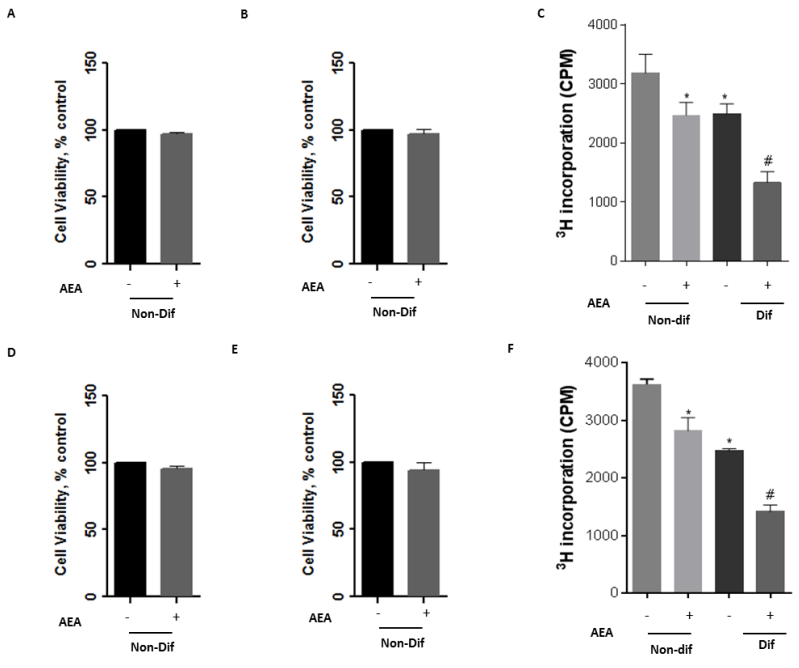
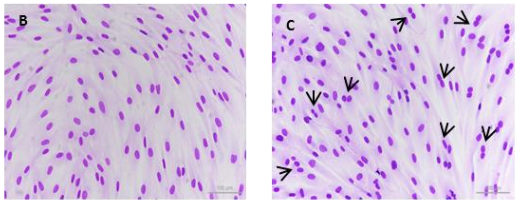


Figure. 3. Effect of AEA in non-differentiated (non-dif) and differentiating cells (dif) viability and proliferation. Differentiation **c**ells were treated with deciduogenic stimulus (cAMP, MPA and E_2) in the absence (control cells) or presence of AEA (10 μM). Cell viability and proliferation were assessed by MTT and ^{3}H-timydine incorporation assay, respectively. AEA did not affect non-differentiated (A) and of differentiating (B) St-T1b cells viability. In St-T1b cell line AEA-inhibits cell proliferation in both cell types (C). AEA did not affect non-differentiated (A) and of differentiating (B) HdF cells viability. AEA decreased DNA synthesis in non-differentiated and in differentiating HdF cells (F). (*p < 0.05 vs. Control; #p < 0.05 vs. Dif).

A

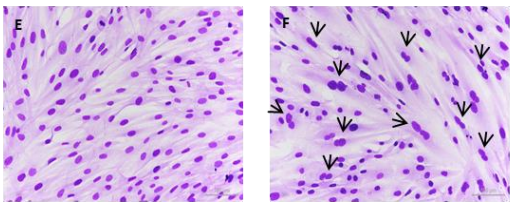
	Non dif	Non dif (AEA)	Dif	Dif (AEA)	AEA + AM281
G0/G1	65,05 ± 0,58	62,13 ± 0,02	70,51 ± 0,20 *	55,32 ± 1,74 #	67,20 ± 1,07 a
S	5,45 ± 0,66	3,49 ± 0,02	2,00 ± 0,15	4,29 ± 0,21	4,63 ± 0,43
G2/M	30,16 ± 1,04	34,38 ± 0,06 *	26,91 ± 0,50	40,40 ± 1,12 #	28,17 ± 1,44 a
2n	94,24 % ± 1,02	90,91 % ± 0,95 *	95,25 % ± 0,60	88,26 % ± 1,87 #	89,00 % ± 1,39 #
> 2n	5,76 % ± 0,66	9,10 % ± 1,02 *	4,74 % ± 0,20	11,74 % ± 1,90 #	11,00 % ± 1,64 #



Dif Dif (AEA)

D

	Non dif	Non dif (AEA)	Dif	Dif (AEA)	AEA + AM281
G0/G1	84,59 ± 1,48	82,00 ± 0,80	92,15 ± 0,65 *	84,77 ± 1,70 #	90,13 ± 0,52 a
S	6,90 ± 1,34	5,63 ± 0,49	1,63 ± 0,42 *	1,88 ± 0,41	1,83 ± 0,07
G2/M	8,51 ± 0,40	12,37 ± 0,95 *	6,22 ± 0,52	13,38 ± 1,48 #	8,04 ± 0,54 a
2n	98,73 % ± 0,29	96,02 % ± 0,36	98,68 % ± 0,44	96,67 % ± 0,49	96,96 % ± 0,14
> 2n	1,27 % ± 0,41	3,98 % ± 0,65 *	1,32 % ± 0,39	3,33 % ± 0,64 #	3,04 % ± 0,14 #



Dif Dif (AEA)

Figure 4. Impact of AEA on cell cycle progression by flow cytometry. In St-T1b cell line, AEA led to a G_2/M retention and appearance of bi-nucleated cells (A), as shown in Giemsa staining of differentiated (B) compared to differentiated AEA-treated St-T1b cells, which present bi-nucleated cells (arrows) (C). In HdF cells, it was also observed a significant arrest at G2/M phase and the accumulation of bi-nucleated cells in AEA-treated cells (D). This effect was also detected in Giemsa staining when comparing differentiated cells (E) and differentiated AEA-treated cells (F). In both cell models, pre-incubation with CB1 antagonist, AM281, prevented cell cycle arrest at G2/M, and induced retention at G0/G1, similar to decidualizing cells. Arrows indicate the presence of bi- and multi-nucleated cells. (*p < 0.05 vs. Control; #p < 0.05 vs. Dif; ap < 0.05 vs. Dif (AEA).

In contrast to St-T1b, approximately 6% of non-differentiated HdF cells were > 2n. In addition, it was observed that pre-incubation with CB1 receptor antagonist did not prevent the appearance of bi-nucleated cells in both cell models. To clarify the mechanism underlying AEA-induced retention at G_2/M, the expression of cyclin B1, a key regulator of G_2/M transition was investigated by Western blot. As shown in Fig.5 A and B, in AEA-treated St-T1b and HdF cells respectively, cyclin B1 protein levels were not altered neither in non-differentiated nor in cells undergoing decidualization. To further explore the signalling pathway underlying AEA-induced effect, Akt activation was investigated by Western blot. AEA prevented Akt phosphorylation in both non-differentiated and differentiating cells compared to respective controls, in both cell models (Fig. 5 C, D). In addition, as in rats, it has been suggested that mitochondria play a crucial role in decidual polyploidization [14], the impact of AEA in mitochondrial transmembrane potential ($\Delta\Psi m$) was studied by flow cytometry on non-differentiated and in cells undergoing differentiation. However, in both cell types and in St-T1b (Fig 6 A) and HdF cells (Fig 6 B), AEA did not induce any alteration in the $\Delta\Psi m$.

Anandamide interferes with cell differentiation

To explore the role of AEA upon the differentiation process, both St-T1b and HdF cells were induced to differentiate in the absence or presence of AEA (10 µM) for 6 d, and mRNA expression of *IGFBP-1* and *PRL* genes was analysed by qRT-PCR. As expected, mRNA levels of both genes were significantly higher in decidualizing cells compared to non-differentiated cells. However, as shown in Fig. 7, the transcript levels of both decidual markers were significantly decreased in cells induced to differentiate in the presence of AEA, to similar levels of those found in non-differentiated cells.

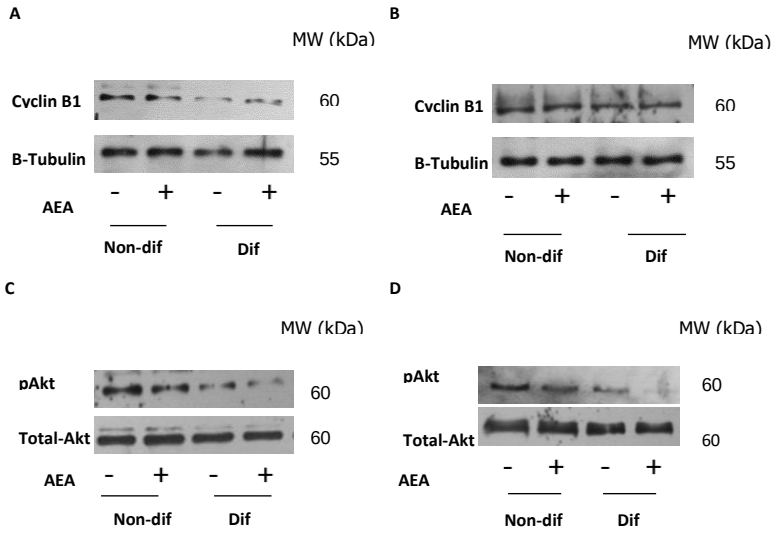


Figure 5. Effect of AEA on Cyclin B1 and Akt protein levels by Western blot. Cyclin B1 expression was not altred by AEA-treatment in both non-differentiated and cells undergoing differentiation in St-T1b cell line (A) and HdF cells (B). However, AEA prevented the activation of Akt in both non-differentiated and cells undergoing decidualization in St-T1b cell line (C) and HdF cells (D).

To investigate the influence of cannabinoid receptors, cells were exposed to the differentiation stimulus in the presence of AEA (10 µM) and pre-incubated with a CB1 antagonist (AM281) or a CB2 antagonist (AM630). In both ST-T1b (Fig. 7 A) and HdF (Fig. 7 B) cells, pre-incubation with AM281, significantly counteracted the AEA-reduction on decidual markers gene expression to levels similar of control decidualizing cells. The contribution of CB2 in the case of HdF was also studied, although the expression of the genes encoding the decidual markers were not affected.

A

	Non-dif	Non-dif (AEA)	Dif	Dif (AEA)
Viable	97,45 ± 0,40	97,21 ± 0,23	96,35 ± 0,20	97,14 ± 0,18
Δψm loss	2,55 ± 0,40	2,79 ± 0,23	3,65 ± 0,20	2,86 ± 0,19

B

	Non-dif	Non-dif (AEA)	Dif	Dif (AEA)
Viable	97,00 ± 0,33	96,83 ± 0,33	98,01 ± 0,28	98,11 ± 0,31
Δψm loss	3,00 ± 0,33	3,17 ± 0,33	1,99 ± 0,28	1,89 ± 0,30

Figure 6. Effect of AEA on mitochondrial transmembrane potential (ΔΨm). Neither in St-T1b cell line (A) nor in HdF cells (B) AEA-treatment affect ΔΨm.

Discussion

Decidualization involves extensive stromal cell proliferation and differentiation, which is followed by programmed cell death, although the underlying molecular mechanisms remain unclear. Endocannabinoids (eCBs) are novel lipid mediators and may be important players in the molecular network composed by hormones, cytokines and growth factors that orchestrates human decidualization. It is known that AEA mediates several cellular events ranging from cell proliferation, differentiation and apoptosis, dependent on the biological system/environment and cellular context [6]. Nevertheless, its role upon the human decidualization is not yet disclosed. In rats, it was previously described that the metabolic enzymes expressed in decidual cells are the main controllers of eCB levels throughout pregnancy [15]. In addition, AEA induces apoptosis in rat primary decidual cell cultures [9, 16] and interferes with decidualization process [10]. Herein it was proposed a novel role for AEA as a modulator of human decidualization, through an effect on both endometrial stromal cell proliferation and differentiation.

For the first time, in this study, it was characterized, the endocannabinoid system (ECS) in St-T1b and HdF cells, in both non-differentiated cells and in cells undergoing differentiation. In contrast to HdF, St-T1b cells do not express CB2 receptor. In contrast to St-T1b cell line, CB1 expression was up-regulated in HdF decidualizing cells, which is in agreement with a previous study [17]. In this regard, it is possible that the endocrine environment to which HdF cells have been exposed may influence CB1 expression. In addition, it was shown that FAAH expression was up-regulated during the decidualization process, while NAPE-PLD expression remained constant in both cell models. These findings are consistent with the expression of the metabolic enzymes in human endometrium during the menstrual cycle, in which FAAH protein levels are higher in the secretory phase, which corresponds to the differentiation of stromal cells, compared to the proliferative phase [18].

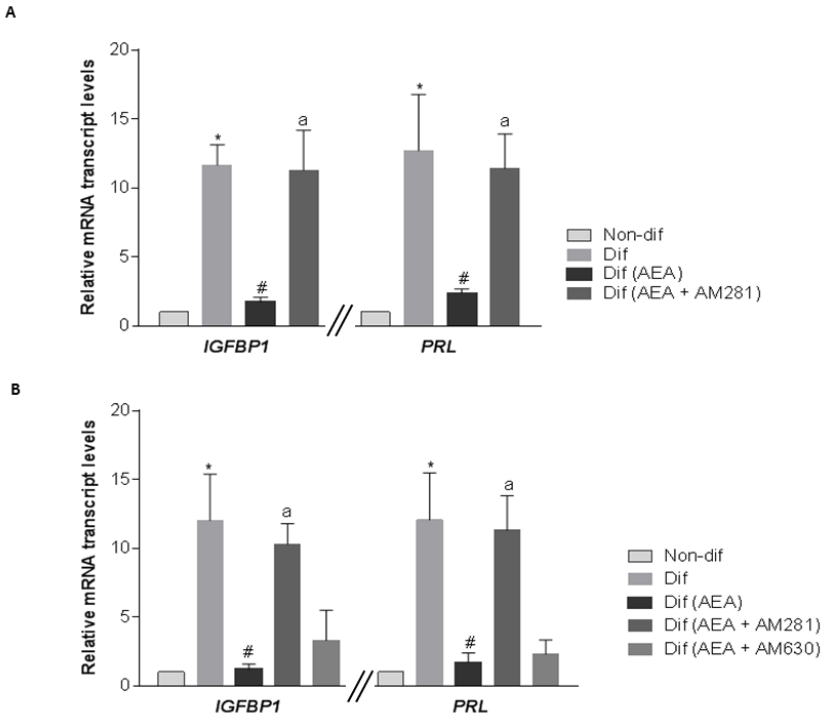


Figure 7. mRNA transcript levels of specific human decidual marker genes. Upon AEA-treatment, *IGFBP-1 and PRL* expression was significantly decreased in St-T1b cell line (A) and HdF cells (B). AEA-induced decreased of mRNA expression of both markers reverted by CB1 antagonist, AM281, in both St-T1b and HdF cells. In HdF, no reversion was observed in the case of AM630, the CB2 antagonist. (*$p < 0.05$ vs. Control; #$p < 0.05$ vs. Dif; a$p < 0.05$ vs. Dif (AEA).

Moreover, our findings further support the recognized role of FAAH, the main AEA degrading enzyme, as a metabolic gatekeeper decreasing AEA uterine levels and promoting the implantation process [7].

In this study, it was also demonstrated that AEA inhibited endometrial cell proliferation supported by a reduction on the synthesis of DNA, cell cycle arrest at the G_2/M phase and accumulation of bi-nucleated cells. The later features were observed in non-differentiated and in cells undergoing decidualization, in both cell models, suggesting that it is not cell specific. In addition, it was found that AEA-treatment did not affect cyclin B1 expression, whereas it prevented phosphorylation of Akt, a signalling pathway involved in cell survival and proliferation [19]. In fact, it has been reported that diminished Akt phosphorylation is associated with inhibition of actin polymerization and consequently cytokinesis failure, appearance of multi-nucleated cells and cell growth inhibition [20]. Moreover, as previously described, the accumulation of bi-nucleated cells and cell cycle arrest at the G_2/M phase may also result from aberrant mitosis and failed cytokinesis, or defects in mitotic spindle checkpoint [21-23]. Therefore, it may be suggested that although cells progress through G_2/M phase, they may fail to undergo cytokinesis, probably due to a direct effect of AEA.

Interestingly, the association of polyploidy with the decidualization process has already been reported. In rodents, endoreduplication (polyploidy) of endometrial stromal cells is well established [24-26]. In fact, it has been reported that decidual polyploidy develops as mono- or bi-nucleated cells due to blockage in G_2/M phase of the cell cycle, as a result of a mitotic cycle without cytokinesis [27]. Moreover, decidua with insufficient polyploidy limits implantation [26, 28], whereas aberrant decidual polyploidy was also shown to be detrimental to this process [29]. In human decidualization, the presence of bi-nucleated cells has been reported by Tang *et al* (1993) [30], and is a topic of increasing recognition in the literature, however their significance has never been addressed. Nevertheless, in the conditions used, AEA-induced bi-nucleation in both non-differentiated and in cells undergoing decidualization, in a mechanism independent of CB1 receptor activation.

Decidualization is a key event for successful implantation, in which endometrial stromal cells cease to proliferate and undergo a differentiation process into decidual cells, being the latter induced by an increase in intracellular cAMP levels. In this study, it was demonstrated that AEA significantly decreased the decidual markers transcript levels, *PRL* and *IGFBP-1*, upon *in vitro* decidualization, in a mechanism dependent on

CB1 activation. Since CB1 activation is known to inhibit adenylate cyclase, it is likely that the AEA inhibitory effect on decidualization is mediated through CB1-induced decrease in cAMP production. In addition, it was shown that AEA prevented cell cycle arrest at G_0/G_1 phase during *in vitro* decidualization, which may hamper cell differentiation. Reinforcing the involvement of CB1 receptor on the inhibitory effect caused by AEA, it was shown that the pattern of cell cycle distribution of cells treated with a selective CB1 antagonist (AM281) plus AEA was similar to the untreated cells undergoing differentiation. Thus, our findings suggest that AEA may be a negative modulator of human stromal cell differentiation. Kessler *et al* [17] previously showed that WIN-55-12, a full CB1 agonist, inhibited decidualization through CB1 by triggering an apoptotic pathway. However, in our conditions, AEA-effect was not accompanied by cell death. Despite in rats it has been suggested that mitochondria may play a role in polyploydization, no alterations in the mitochondrial transmembrane potential were observed.

Understanding the molecular signature that underlies the role of AEA on endometrial stromal cells further elucidates the cellular and molecular basis that governs decidualization. Two major breakthroughs are highlighted in the present study. On one hand, AEA inhibits endometrial stromal cell proliferation and leads to the appearance of bi-nucleated cells, which may consequently impair stromal cell differentiation. On the other hand, AEA also act as a negative modulator of endometrial stromal cell differentiation through a CB1 dependent mechanism. In women, AEA levels fluctuate during menstrual cycle, reaching high levels at the proliferative phase and low levels at the secretory phase, which corresponds to the beginning of the differentiation process [15, 31]. Moreover, it is widely accepted that AEA levels are strictly regulated mainly by FAAH activity. Altogether, our findings corroborate that a balance tone of AEA uterine levels is crucial for human decidualization, and thus elevated levels of this eCB, due to cannabinoids consumption or to FAAH deregulation, may impair this process. We also suggest that AEA may be a central tie bridging both steps of the decidual program, stromal cell proliferation and differentiation. Decidualization is a remarkable feature of human reproduction. It is known that impaired decidualization process is associated with a variety of reproductive disorders, ranging from infertility, recurrent miscarriage, utero-placental interaction disorders. Nowadays, failure of the endometrium to achieve a receptive state is consider as a major cause of infertility as well as the rate-limiting step in assisted reproductive technology. Therefore, unveiling the molecular mechanisms that underpin decidualization process is of great importance to improve reproductive outcome.

Acknowledgments

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Supplemental Material

Table 1. Primer sequences for control and target genes. Detailed description of all genes tested, primer pairs' sequences and Q-PCR conditions.

Symbol	Accession Number	Description	Primers (5'-3')	Amplification conditions[1)]	Tm (ºC)	Reference
IGFBP1	NM_000596.2	Homo sapiens insulin-like growth factor binding protein 1	S: GAGATAACTGAGGAGGAG AS: CCAAAGGATGGAATGATC	95ºC, 03 sec 59ºC, 30 sec 72ºC, 90 sec	78	[12]
PRL	NM_001163558.2	Homo sapiens prolactin	S: CTTCATTCCAGAAGTACCCT AS: TCTTTCCCAGATATTGGCTT	95ºC, 03 sec 60ºC, 30 sec 72ºC, 90 sec	80	
CB1	NM_001160259.1	Homo sapiens cannabinoid receptor 1	S: CTTCCCACAGAAATTCCC AS: TACCTTCCCATCCTCAGA	95ºC, 03 sec 62ºC, 30 sec 72ºC, 90 sec	87	[8]
CB2	NM_001841.2	Homo sapiens cannabinoid receptor 2	S: CGTGGCTGTGCTCTATCTGA AS: ATCTCGGGGCTTCTTCTTTT	95ºC, 03sec 62ºC, 30 sec 72ºC, 90 sec	87	[8]
FAAH	NM_001441.2	Homo sapiens fatty acid amide hydrolase	S: GGCCGTCAGCTACACTATGC AS: ATCAGTCGCTCCACCTCCC	95ºC, 03 sec 59ºC, 30 sec 72ºC, 90 sec	87	[8]
NAPE-PLD	NM_001122838.1	Homo sapiens N-acyl phosphatidylethanolamine phospholipase D	S: AGATGGCTGATAATGAGAA AS: TTCTCCTCCCACCAGTC	95ºC, 03 sec 58ºC, 30 sec 72ºC, 90 sec	72	
GAPDH	NC_000012.12	Homo sapiens glyceraldehyde-3-phosphate dehydrogenase	S: AGAACATCATCCCTGCCTC AS: GCCAAATTCGTTGTCATACC	95ºC, 03 sec 55ºC, 30 sec 72ºC, 90 sec	78	[8]

•Cycling conditions were as follows: an initial step at 95°C 3 min for enzyme activation in all cases, followed by up to 40 cycles of denaturation, annealing and primer extension as described.
*The primers for Igfbp1, Prl and Nape-pld were designed in-house using Primer Premier (version 5.0 software; Premier Biosoft International, Palo Alto, CA; wwpremierbiosoft.com).

Manuscript IV:

Anandamide interferes with human endometrial stromal derived-cell differentiation: an effect dependent on inhibition of COX-2 expression and PGE_2 release

Almada M., Cunha S., Fonseca B.M., Amaral C., Piscitelli F., Di Marzo V., Correia-da-Silva G., Teixeira N.

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Anandamide interferes with human endometrial stromal derived-cell differentiation: an effect dependent on inhibition of COX-2 expression and PGE_2 release

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Abstract

The human endometrium undergoes cyclical growth, differentiation and regression periods throughout the reproductive life. The process in which endometrial stromal cells proliferate and differentiate into decidual cells, named decidualization, prepares a receptive endometrium for implantation. Prostaglandins (PGs) and endocannabinoids (eCBs) are crucial mediators of this process. We have recently reported that the eCB anandamide (AEA) interferes with rat stromal cell differentiation, and on the other hand, PGs are also crucial for decidualization. Therefore, in this study, we analysed AEA levels, both in non-differentiated and in decidualizing human endometrial stromal cells by liquid chromatography-mass spectrometry (LC-MS), and investigated the impact of AEA on PG release and COX-2 expression in human endometrial stromal derived-cell differentiation. For that, an ultra-performance LC-MS/MS method to measure prostaglandin E_2 (PGE_2) and prostaglandin $F_{2\alpha}$ ($PGF_2\alpha$) in biological samples was developed and validated. We demonstrate that AEA levels in decidualizing cells are lower than in non-differentiated cells, whereas PGE_2 levels and COX-2 expression are up-regulated. Thus, low AEA levels may be essential for the onset of decidualization. On the contrary, in AEA-treated cells undergoing decidualization, a decrease of COX-2 protein levels and PGE_2 production, in a manner dependent on cannabinoid receptor 1 activation was observed. Overall, these findings suggest that a deregulation of the intricate network that drives cell differentiation may compromise pregnancy and fertility. It is clinically relevant to understand the mechanisms that influence eCB and PG levels in the endometrium because they may shed light on the sequence of events that lead to a successful pregnancy.

Keywords: Endocannabinoids, Prostaglandins, Decidualization, UPLC-MS/MS

Abbreviation list: AA – arachidonic acid; AEA - anandamide, cAMP - 8-Bromoadenosine 3',5'-cyclic monophosphate; CB1 – cannabinoid receptor 1; COX-2 - cyclooxygenase-2; E2 – estradiol; eCBs – endocannabinoids; electrospray ionization source – ESI; fatty acid amide hydrolase – FAAH; human decidual fibroblasts – HdF; human-immortalized endometrial stromal cell line - St-T1b; insulin-like growth factor-binding protein 1 - IGFBP-1; liquid chromatography-mass spectrometry - LC-MS; limit of quantification – LOQ; lower limit of detection – LOD; medroxyprogesterone acetate – MPA; prostaglandin E2 - PGE2; prostaglandin F2α - PGF2α; prostaglandin-ethanolamides - PG-EAs; relative standard deviation - RSD; ultra-performance liquid chromatography-mass spectrometry - UPLC-MS/MS;

Introduction

Decidualization denotes the transformation of the endometrial stromal compartment, into specialized decidual cells, which is essential to accommodate pregnancy (1). In humans, it is widely accepted that a manifold of paracrine and autocrine cues, as growth factors, cytokines and transcription factors, drive decidualization (2). Although major advances are being put forward into the identification of the (bio)molecular repertoire that orchestrates the receptive endometrium, this is not yet clarified. Amongst other lipid messengers, prostaglandins (PGs) and endocannabinoids (eCBs) are considered as pivotal mediators.

Prostaglandins, derived from cyclooxygenase-2 (COX-2), are believed to play an important role in regulating decidualization, angiogenesis, vascular remodelling, trophoblast invasion and extracellular matrix remodelling during implantation (3). In rats, COX-2 expression is mostly restricted to implantation sites, and COX-$2^{-/-}$ mice are infertile, with defective implantation and decidualization (4,5). In humans, deregulated PG signaling has recently been correlated to poor endometrial receptivity, infertility and uterine malignancies (6,7).

Like PGs, endocannabinoids (eCBs) are endogenous mediators derived from membrane phospholipids. They regulate several cellular events, such as cell proliferation, differentiation and survival, with different outcomes depending on the molecular targets and cellular context (8). Over the last years, eCBs have been identified as novel players in the complex molecular network that coordinates implantation and decidualization (9). In rats, it was shown that anandamide (AEA)

controls decidual turnover (10) and, recently, we have demonstrated that AEA interferes with rat decidual phospholipid membrane composition (11). The tight regulation of AEA levels throughout the differentiation process and implantation is of crucial importance, as aberrant eCB signaling may lead to spontaneous miscarriage and poor pregnancy outcome (12,13). Anandamide (AEA) and other known eCBs act primarily on G-protein-coupled cannabinoid receptors 1 and 2 (CB1 and CB2), and are metabolized by specific enzymes, which altogether form the eCB system (ECS). Fatty-acid amide hydrolase (FAAH) metabolizes AEA into arachidonic acid (AA) and ethanolamine. The former provides a source for PGs production by COX-2. Furthermore, AEA is a direct substrate of COX-2 oxidative metabolism, producing prostaglandin-ethanolamides (PG-EAs) (14). Recently, it was recently shown that in rat decidual cells, AEA-induced cell death through COX-2 oxidative metabolism (15). On the other hand, in rats, it has also been reported that AEA limits the progression of the stromal differentiation process and that PGs are important mediators for the differentiation process (16). The regulatory PG function in early human pregnancy events is not yet clear. Although the evidence underlying the crosstalk between eCBs and PGs are particularly apparent upon decidualization, little is known about this network. Therefore, in this study, we aimed to decipher the effect of AEA on COX-2 expression and PG production during *in vitro* decidualization of human endometrial stromal derived-cells.

Material and methods

St-T1b cell line culture

The human endometrial stromal cell line (St-T1b) was kindly supplied by Dr. Birgit Gellersen from Endokrinologikum Hamburg, Hamburg, Germany. This cell line was obtained from uterine biopsy samples at the time of hysterectomy from benign gynaecological disorders and immortalized with human telomerase reverse transcriptase (hTERT) (17). These cells were maintained at 37 ºC with 5% CO_2 in DMEM/F-12 medium supplemented with 10% charcoal-stripped fetal bovine serum (CT-FBS), 1 nM 17-β-estradiol (E_2), 1 µg/ml insulin and 2% penicillin-streptomycin-amphotericin B. Cells from 3 to 10 subpassages were used in these studies.

Primary cultures of human decidual fibroblast (HdF)

The protocol for the isolation of cells from term decidua was based on Richards et al, 1995 (18). Caesarean or vaginal delivery normal human placentas (38–40 weeks of gestation) from Caucasian women, living in Porto region and aged 24–36 years old, were obtained from Centro Materno-Infantil do Norte, Porto. All the procedures using term placentas were performed according to the Ethical committee of Centro Materno-Infantil do Norte. The decidua basalis was scraped from the chorionic membrane, dissected into small pieces and enzymatically digested in PBS with collagenase (1 mg/ml) for 1 h at 37 ºC with gentle shaking. The suspension was then centrifuged at 260 *g* for 6 min. The pellet was resuspended with ammonium chloride (0.8 %) solution for red blood cells lysis, at 37 ºC. The resulted suspension was filtered through a 40 µm nylon mesh and centrifuged at 260 *g* for 10 min. The isolated cells were maintained at in 37 ºC with 5% CO_2 in DMEM/F-12 medium supplemented with 10% CT-FBS, 1 nM E_2, 1 µg/ml insulin and 2% penicillin-streptomycin-amphotericin B. After 24 h, the non-adherent cells were removed and the cells were grown to confluence and sub-cultured. By subpassage 3, essentially all the cells were proliferating fibroblasts. Cells from 3 to 10 subpassages were used in these studies. For each assay, it was used decidual fibroblasts from five different placentas and studies were performed independently (n=5).

Differentiation treatment

In vitro differentiation was induced in minimal medium MM1 (DMEM/F-12 medium supplemented with 2% CT-FBS and 2% penicillin/streptomycin), containing 1 µM medroxyprogesterone acetate (MPA; Sigma-Aldrich, St. Louis, MO, USA), 10 nM estradiol (E_2, Sigma-Aldrich, St. Louis, MO, USA), and 0.5 mM of 8-Bromoadenosine 3',5'-cyclic monophosphate (cAMP, Sigma-Aldrich, MO, USA) or 0.05 mM cAMP, for St-T1b and HdF, respectively. The differentiation process was induced for 4 or 6 days, and the medium was changed every 2 or 3 days, respectively. Cell differentiation was confirmed by morphological analysis and measurement of insulin-like growth factor-binding protein 1 (IGFBP-1) by ELISA. Cells were treated with the differentiation medium (cAMP, MPA and E_2) in the presence or absence of AEA (10 µM). In order to investigate the role of CB receptors, cells were also pre-incubated with selective CB antagonists, AM251 and AM281 (CB1 antagonists, 1 µM) and AM630 (CB2 antagonist, 1 µM) for 30 minutes before the addition of AEA (10 µM). Non-differentiated cells treated in MM1 in the absence or presence of AEA (10 µM) were used as a control.

Measurement of IGFBP-1 in cell culture medium by ELISA

IGFBP-1, one of the main secretory products of human decidual cells, widely used as a biochemical marker of the decidualization process, was measured in culture medium from endometrial stromal cells by ELISA kits (BioVendor, Laboratorni medicina, Brno, Czech Republic) according to the manufacturer's instruction, and normalized to total DNA levels. The sensitivity of this assay was 0.2 ng/ml.

Western blot

St-T1b cells and HdF (both non-differentiated and undergoing differentiation) were cultured and treated as described for 4 days. Cell extracts were prepared in a potassium phosphate buffer (20 mM Tris-HCl, 100 mM of NaCl, 1mM of EDTA) containing a cocktail of protease and phosphatase inhibitors (P8340, Sigma-Aldrich, St. Louis, USA). Protein concentrations were measured by Bradford assay. Samples (80 µg) were run on 10 % SDS polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were then incubated with goat COX-2 antibody (sc-1745, Goat, Santa Cruz Biotechnology, CA), at 4 °C overnight. Membranes were then washed and incubated with Rabbit anti-goat IgG horseradish peroxidase–conjugated antibody (sc-2922, Santa Cruz Biotechnology, CA) and detected by enhanced chemiluminescence. β-tubulin (h-235, Rabbit, Santa Cruz Biotechnology, CA) was used as a loading control. The signal intensity of each band was quantified by densitometry (BIO-PROFIL Bio-1D++, Vilber Lourmat, France).

LC-APCI-MS method for AEA quantification

For AEA quantification, cell pellets from non-differentiated and from cells differentiated for 6 days were collected and stored at -80 ºC. Extraction, purification and quantification of AEA were performed as previously described (19). Cell pellets were homogenized and AEA extracted with acetone containing internal deuterated standards ($[^2H]_8$ AEA) for AEA quantification by isotope dilution LC-MS. The lipid-containing organic phase was dried down, weighed, suspended in methanol and pre-purified by open bed chromatography on silica gel. The AEA-containing fraction was obtained by eluting the column with 90:10 (v/v) chloroform/methanol, and used for AEA quantification by liquid chromatography-atmospheric pressure chemical ionization-mass spectrometry (LC-APCI-MS), using selected ion monitoring at M^{+1} values. Results are expressed in pmol/mg and were normalized to total amount of lipid content.

UPLC-MS/MS based protocol for PGE_2 and $PGF_{2\alpha}$ quantification

Sample preparation

To the homogenized sample (2 ml) in a 4 mL screw-capped glass vial, it was added 25 µL of d_4-PGE_2 (IS, 1 mg/L) and 2 mL ethyl acetate, 0.8 g $MgSO_4$ and 0.2 g NaCl. After shacking for 5 min, the sample was centrifuged at 3500 *g* for 5 min. The upper-layer phase was transferred to a new vial, and evaporated to dryness under a gentle stream of nitrogen. The sample was resuspended in 100 µL of the mobile phase and injected in the UPLC-MS/MS system.

UPLC-MS/MS analysis

Separation and quantification of the target analytes were performed by using a liquid chromatography Acquit UPLC system interfaced to a triple quadrupole mass selective detector Micromass Quattro micro API™ (Waters, Milford, MA, USA). The injection volume was set at 10 µL. The chromatographic separation was carried out with a BEH C18 column (1.6 µm, 2.1 mm x 150 mm (i.d.)) from ACQUITY UPLC, at a flow rate of 0.20 mL min^{-1}. The mobile phases were (A) 5% ammonium acetate (5 mM) in ultra-pure MilliQ water and (B) MeOH (UPLC grade, VWR, Radnor, PA) in an isocratic gradient. Mass spectrometry analysis was performed with an electrospray ionization (ESI) source in the negative (ESI-) ion mode for all the analytes. Nitrogen was used as the nebulizer gas. The optimum MS parameters were: capillary, 3.00 kV; extractor, 2 V; RF Lens, 0.5 V; Source Temperature, 150ºC; Desolvation Temperature, 350ºC; Desolvation Gas Flow, 350.0 L/h; Cone Gas Flow, 60.0 L/h; LM Resolution, 13.0; Ion energy, 1.0; Entrance, 1; Exit, 2; Multiplier, 650. All analyses were done in multiple reaction monitoring (MRM) mode. The UPLC-MS/MS settings and ESI source parameters were optimized by manual infusion of standards with a syringe pump and acquisition of the respective daughters' spectrum after collision in the quadrupole. The optimized parameters are summarized in Table 1. The analytical method validation was performed according to the guidelines of the FDA (U.S.F.D.A.—FDA, (2001)). The parameters studied were linearity, limit of detection (LOD), limit of quantification (LLOQ), precision, accuracy and recovery.

Statistical analysis

Statistical analysis was carried out by Student's *t*-test to compare difference between the control and the test groups. PGE_2 quantification statistical analysis was carried out by t-student and One-way ANOVA and followed by Tukey test (GraphPad PRISM v. 6.0, GraphPad Software, Inc., San Diego, CA, USA). The results are the mean of at

least five independent experiments performed in triplicate. Data were expressed as mean ± SEM and differences were considered to be statistically significant at $p < 0.05$.

Results

AEA levels in non-differentiated and decidualizing endometrial stromal cells

As expected, secretion of IGFBP-1, the main secretory product of decidual cells, was higher in decidualizing cells compared to non-differentiated HdF and St-T1b cells (Fig. 1 A, B). In cells undergoing decidualization, AEA treatment caused a significant decrease of IGFBP-1 release, to levels similar of those found in non-differentiated cells.

To explore the role of AEA in the endometrial environment, its levels were measured by LC-APCI-MS, in both non-differentiated and decidualizing cells. AEA levels were lower in stromal cells undergoing differentiation than in non-differentiated cells. In HdF cells (Fig. 1 C), endogenous AEA levels were 14.3 pmol/mg, while they significantly decreased to 4.9 pmol/mg in decidualizing cells. In non-differentiated St-t1b cells we found 5.2 pmol/mg of AEA, as compared to 1.82 pmol/mg in cells undergoing differentiation (Fig. 1 D). Interestingly, in both cell types, AEA levels were 3-fold lower in decidualizing cells as compared to non-differentiated cells.

UPLC-MS-MS based protocol for PGE_2 and $PGF_{2\alpha}$ quantification/Method Validation

The deprotonated molecules of PGE_2 and $PGF_{2\alpha}$ were detected at m/z 351 and 354, respectively during negative ion electrospray mass spectrometry (Fig. 2). Collision induced dissociation of the [M-H]- ions, producing abundant fragments m/z 333, 315, 271, and 263, 247 and 193, respectively for PGE_2 and $PGF_{2\alpha}$. The product ion tandem mass spectra are shown in Figure 2 and Table 1. The most abundant product ion of each analyte, PGE_2 and $PGF_{2\alpha}$, was selected for the quantitative analysis, and corresponded to m/z 271, and 247 respectively. The corresponding transition of m/z 355 to 275 was monitored for the deuterated internal standard (IS) d4-PGE_2 (Fig. 2 E, F). The chromatograms of PGE_2, $PGF_{2\alpha}$ and d4-PGE_2 are shown in Fig 2 respectively. The linearity of the method was evaluated by analyzing different concentrations between 0.05 nM and 100 nM for PGE_2 and between 1 nM and 100 nM for $PGF_{2\alpha}$ (Fig. 3).

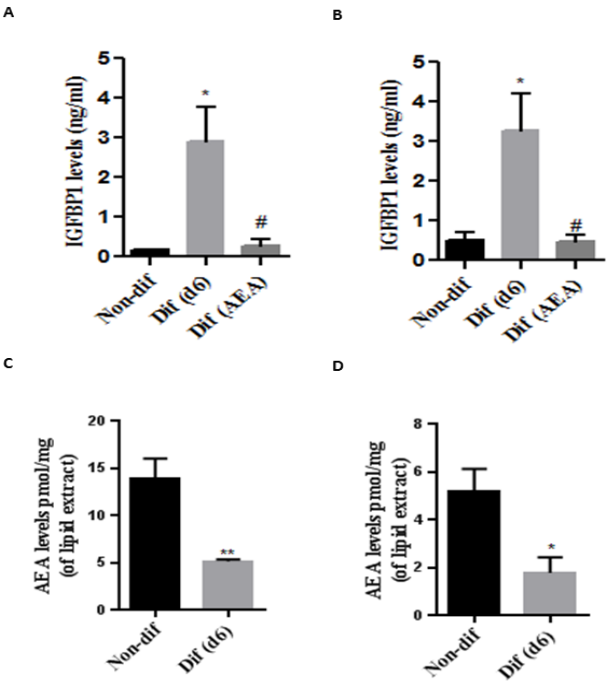


Figure 1 – IGFBP-1 levels in cell culture medium by ELISA and AEA levels in endometrial stromal cells by LC-APCI-MS. IGFBP-1 levels in the cell culture medium of non-differentiated cells (non-dif), decidualizing cells (dif 6d) in the presence or absence of in HdF cells (A) and the St-T1b cell model (B). (*$p < 0.05$ vs. Control; #$p < 0.05$ vs. Dif 6d). C) Anandamide levels in non-differentiated and decidualizing HdF cells by LC-APCI-MS. A 3-fold decrease of AEA levels was observed in decidualizing cells. D) AEA levels were significantly lower in St-T1b decidualizing cells compared to non-differentiated. (*$p < 0.05$ vs. Control; ** $p < 0.01$ vs. Control; #$p < 0.05$ vs. Dif 6d). (HdF, Human decidual Fibroblasts; St-T1b, telomerase-immortalized human endometrial stromal cell line; non-dif, non-differentiated cells; dif 6d, decidualizing cells after 6d of differentiation treatment).

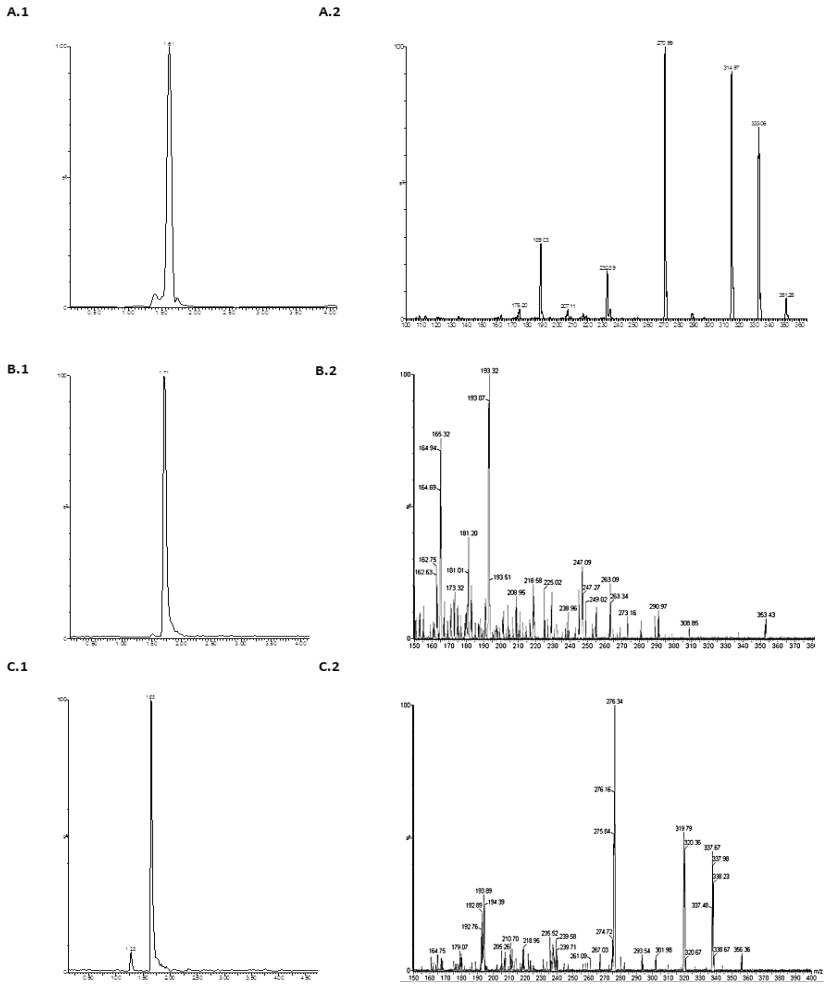


Figure 2 – Negative ion UPLC-MS/MS chromatograms. Total ion chromatograms (TIC) of standard solutions of PGE_2 (A.1), $PGF_{2\alpha}$ (B.1) and d_4-PGE_2 (C.1), and spectrum of PGE_2 (A.2), $PGF_{2\alpha}$ (B.2) and d_4-PGE_2 (C.2).

Calibration curves were constructed by plotting the analyte/IS (ratio of analyte, ARE) obtained against the concentration of each analyte. The calibration curves were linear for both PGs, presenting a coefficient of determination (r2) of 0.9994 and 0.9982, for PGE_2 and $PGF_{2\alpha}$, respectively (Table 2). The linearity of the method was tested at least five times using the matrix-matched calibration (cell culture medium).

The lower limit of detection (LOD, defined as a signal-to-noise of 3:1), was 0.05 nM for PGE_2 and 0.5 nM for $PGF_{2\alpha}$ (Table 2). The limit of quantification (LOQ, which corresponds to the lowest concentration measured with acceptable accuracy, precision and relative standarddeviation (%RSD) <20%), was for PGE_2 and $PGF_{2\alpha}$ respectively, 1 nM and 5 nM (Table 3).

The intra- and inter-assay precision was determined using samples spiked with three different concentrations 1 nM, 0.2 µM and 2 µM of each PG. Intra-assay precision was evaluated by performing the extraction and analysis of six spiked samples in the same day while inter-assay precision was determined by analysis of triplicate spiked samples in three different days for a period of 3 weeks. The relative standard deviation (%RSD) was below 12% for both intra- and inter-assay precision (Table 3 and Fig. 3 A, B), which is within the normal criteria accepted for bio-analytical methods validation. These results indicate that this method showed excellent accuracy and precision. The recovery of PGE_2 and $PGF_{2\alpha}$ from the cell culture medium was determined at different concentrations by comparing the peak areas of spiked and processed cell culture medium with the corresponding standard solutions analyzed without extraction. Extraction efficiency was evaluated considering the following formula: R = (SS-NS) x 100 / As (where SS is the value obtained from spiked sample, NS from not spiked sample and As the amount added). Extraction efficiency was higher than 98% for each analyte (Table 4 and Fig. 3 C, D) suggestive of a good efficiency of the extraction procedure.

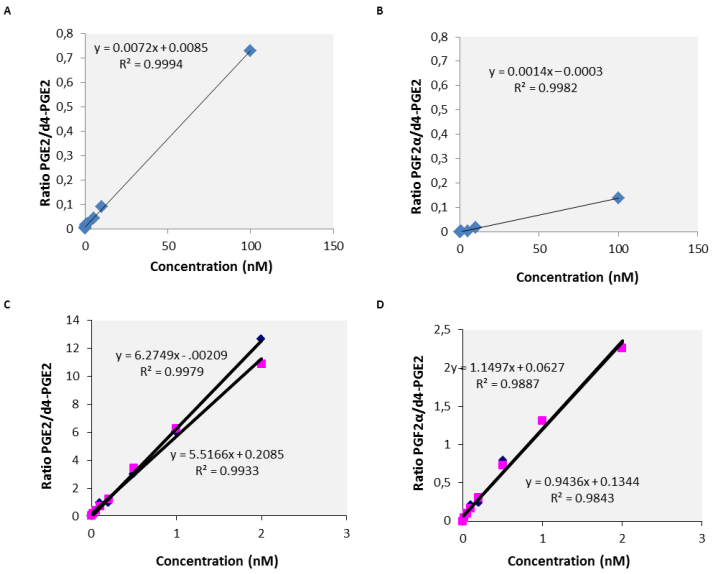


Figure 3 – Linearity and Recovery efficacy of the UPLC-MS/MS method. Linearity of the method evaluated by calibration curves of standard solution of PGE_2 (A), $PGF_{2\alpha}$ (B). Calibration curves were constructed by plotting the analyte/ARE ratio against the concentration of each analyte. r^2 of 0.9994 and 0.9982 for PGE_2 and $PGF_{2\alpha}$ was obtained, respectively. The recovery of PGE_2 (C) and $PGF_{2\alpha}$ (D) from cell culture medium was determined at different concentrations by comparison of the peak area of the spiked and processed samples with the corresponding standard solution analysed without extraction.

AEA-induced effect on PGE_2 levels

PG levels were measured in the culture medium of non-differentiated cells and of cells undergoing differentiation, using both cell models (Fig. 4). As expected, PGE_2 was significantly increased in decidualizing cells, both in the HdF (9.18 nM) and St-t1b cell line (6.99 nM), as compared to non-differentiated cells (2.99 nM and 1.63 nM, respectively). However, $PGF_2\alpha$ was not detected probably because below the detection limit.

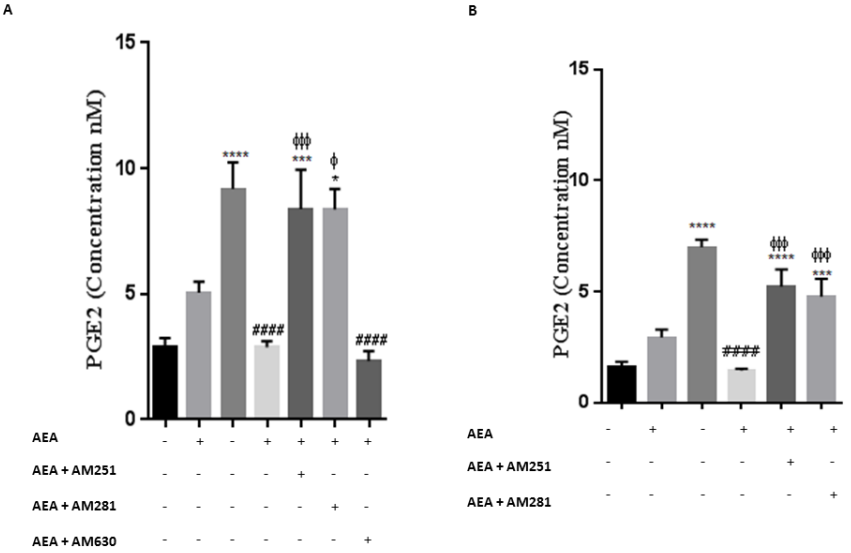
Due to the inverse pattern between AEA and PG levels during decidualization, we hypothesized that decidualization follows a reduction on AEA levels and an increase on PG levels. Therefore, the effect of AEA on PG production was also examined, in non-differentiated cells and during stromal cell differentiation (Fig. 4).

Strikingly, in undifferentiated stromal cells, AEA-treatment induced a similar 1.8-fold increase on PGE_2 levels in both cell types. However, during decidualization, AEA significantly reduced PGE_2 secretion, to levels similar to those found in non-differentiated cells. In HdF cells, PGE_2 decreased from 9.18 nM in decidualizing cells to 2.88 nM in AEA-treated decidualizing cells, which represents a 3-fold decrease (Fig. 4 A, C). In St-T1b decidualizing cells, PGE_2 levels were 6.99 nM and AEA-treatment induced a reduction of almost 5-fold to 1.4 nM.

To explore the involvement of cannabinoid receptors, cells undergoing decidualization were pre-incubated with selective antagonists for either CB1 (AM251 and AM281) or CB2 (AM630), before the addition of AEA. In HdF cells, the treatment with CB1 antagonists increased the levels of PGE_2, to 8.37 nM and 7.42 nM, for AM251 and AM281, respectively. These levels were similar to those found in the untreated differentiated cells (9.18 nM), suggesting a CB1 receptor-dependent effect for AEA (Fig. 5 A, C). Instead, the CB2 antagonist did not reverse the AEA-inhibitory effect, as levels (2.33 nM) were similar to those in decidualized AEA-treated cells. In the St-T1b cell line, CB1 antagonists also significantly counteracted the inhibition of PGE_2 levels (5.22 nM and 4.78 nM) (Fig. 4 B, C). The effect of the CB2 antagonist was not investigated since, as described previously, this receptor was not detected in this cell line (20).

Correlation between PGE_2 concentrations and COX-2 protein expression

As expected, decidualizing cells express higher levels of COX-2 than non-differentiated cells. AEA treatment did not affect COX-2 protein levels in non-differentiated cells (Fig. 5). However, in both cell types, when undergoing decidualization, AEA-induced a significant decrease in COX-2 protein levels, which was directly correlated with the reduction of secreted PGE_2. As CB1 antagonists were able to counteract the AEA-inhibitory effect on PGE_2 production, we also investigated the role of CB1 receptors in COX-2 protein expression. Pre-incubation with CB1 antagonists reversed the AEA-inhibitory effect on COX-2 protein levels in the St-T1b cell line, and attenuated it in HdF cells (Fig. 5 C, D).



C

	Non-differentiated cells		Differentiated cells				
	Non-dif	AEA	Dif	AEA	AEA + AM251	AEA + AM281	AEA + AM630
HdF	2.98 ± 0,34	5.04 ± 0,45	9.18 ± 1,0	2.88 ± 0,23	8.37 ± 1,6	7.42 ± 1	2.33 ± 0,38
St-T1b	1.64 ± 0,21	2.94 ± 0,35	6.99 ± 0,35	1.44 ± 0,08	5.23 ± 0,77	4.78 ± 0,8	

Figure 4 – PGE_2 quantification levels by the developed UPLC-MS/MS method. A) PGE_2 measurement in the culture medium of HdF cells. PGE_2 secretion was significantly increased in decidualizing cells. AEA-treatment reduced PGE_2 levels in differentiated cells. Pre-incubation with CB1 antagonists abolished the AEA-inhibitory effect, while CB2 antagonist (AM630) did not affect PGE_2 production, as levels were similar to AEA-treated cells. B) PGE_2 measurement in St-T1b cells. Differentiation treatment significantly increased PGE2 levels, whereas AEA-induced a 4-fold decreased. C) PGE_2 levels measurements in the cell culture medium in both HdF and St-T1b. Results are expressed the mean ± SEM of at least five independent experiments (***$p < 0.001$ vs. Control; **** $p < 0.0001$ vs. Control; #### $p < 0.0001$ vs. Differentiated; ΦΦΦ $p < 0.001$ vs. Dif (AEA); ΦΦΦΦ $p < 0.0001$ vs. Dif (AEA)).

Discussion

The human endometrium is a dynamic tissue that in every menstrual cycle undergoes periods of proliferation, differentiation and programmed cell death. Reproductive disorders, such as implantation failure and infertility, have been linked to poor endometrial receptivity and impaired decidualization (2). Over the last years, the development of lipidomic approaches has enabled the identification of lipid mediator candidates for endometrial receptivity. Of these, endocannabinoids (eCBs) and prostaglandins (PGs) have emerged as key players. Recently, we reported that the eCB AEA limits the process of rat stromal cell differentiation, possibly through COX-2 down-regulation (16). Nonetheless, the potential eCB-PG crosstalk beneath the maternal endometrium was not disclosed. In the present study we suggest that levels of AEA might disrupt COX-2 expression and PG release, with a mechanism dependent on CB1 activation.

On the other hand, we found that AEA decreases the levels of IGFBP-1, suggesting that it may also interfere with the decidualization process.

Although this study uses two *in vitro* models of human endometrial stromal derived-cells, which are nevertheless documented as suitable models for differentiation studies, we hypothesized that the impact of AEA on IGFBP-1 levels may, at least in part, reflect a deregulation of PG metabolism and signaling pathways. We report, for the first time, the AEA levels in non-differentiated and in decidualizing human stromal cells, in both St-T1b and HdF cells. Upon differentiation, AEA reached very low levels in both cell models, suggesting that this eCB may be important for maintaining stromal cells in an undifferentiated state and that its temporal down-regulation may trigger the decidual reaction. Consistently, it has been previously described that AEA plasma levels fluctuate through the menstrual cycle with lowest levels found during the mid-luteal phase, which coincides with the decidualization process (21). Moreover, it is widely accepted that a local tone of AEA in the human endometrium is primarily regulated by FAAH expression, which is the major enzyme responsible for AEA hydrolysis (22).

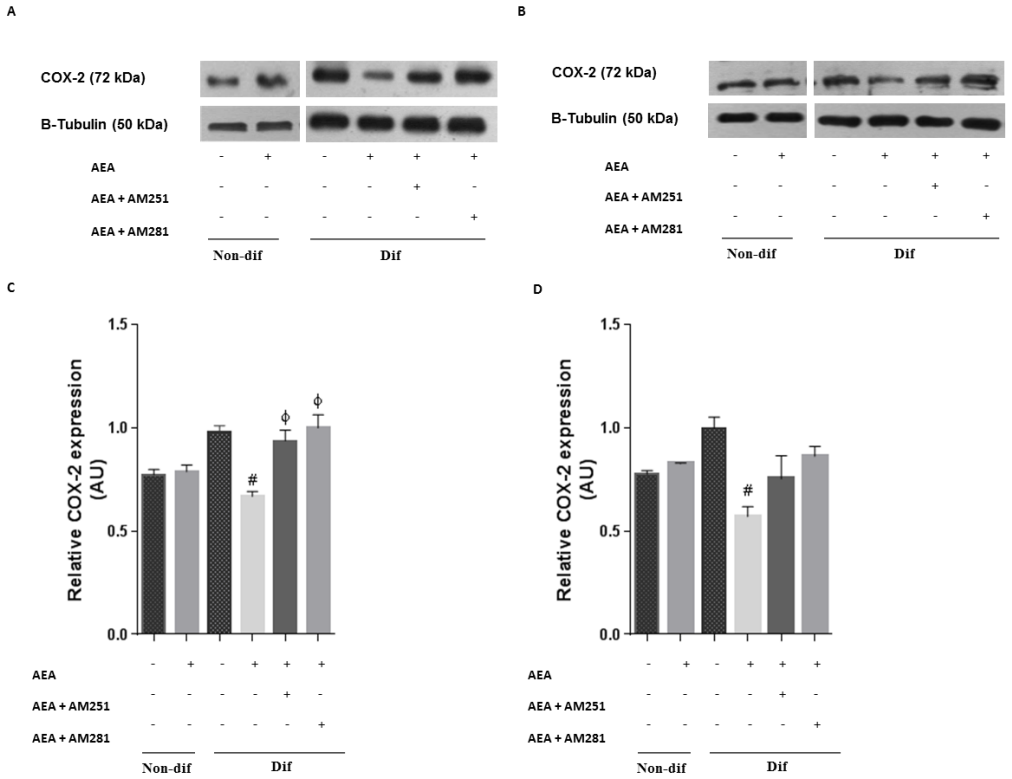


Figure 5 – COX-2 protein levels evaluated by Western Blot. A) In HdF cells, differentiation treatment was accompanied by an up-regulation of COX-2 expression. AEA-treatment decreased COX-2 protein levels; this effect was attenuated by CB1 antagonists. B) Similarly to HdF, in St-T1b cells, COX-2 was up-regulated in differentiated cells, and AEA induced a down-regulation of COX-2, mediated through CB1 receptor. C) Quantification of the COX-2 expression. B-Tubulin was used as a control. (AU) arbitrary units (# $p < 0.05$ vs. Differentiated; Φ $p < 0.05$ vs. Dif (AEA)).

Thus, a deregulation of eCB signaling may lead to inappropriate decidualization and, consequently, compromise embryo implantation and establishment of pregnancy. As previously mentioned, eCBs and COX-2-derived PGs are emergent mediators during early pregnancy. In humans, PGs are elevated during the receptive period (23). In this sense, in rodents, low AEA levels correlate with uterine receptivity (24). In the rat uterus, during the window of the implantation period, lysophosphatidic acid increased FAAH activity, reduced AEA levels and up-regulated COX-2 expression (25). Furthermore, the pattern of COX-2 expression in the pregnant rat uterus suggests a fundamental role during embryo implantation and decidualization (4,5). In animal models, PG signaling has been implicated in increased vascular permeability, stromal decidualization, embryo development and extracellular matrix remodelling during implantation (3). Although, in humans, still little is known about the role of PGs in decidualization. Achache *et al* (7) reported defective endometrial PG synthesis in women with repeated failures of *in vitro* fertilization, and thereby poor receptivity for embryo implantation. The potential use of PGs as biomarkers for endometrial receptivity and early pregnancy failure has increasingly been explored (23). However, limitations in PG measurement by GC-MS and by unselective immunoassays have hampered the thorough profiling of these molecules in human samples, and hence narrowed the translation of knowledge from preclinical studies and animal models into a clinical benefit. LC-MS based methods, relying on their sensitivity, high selectivity and simplicity of sample preparation, have evolved as tools for quantifying PGs (26,27). In the present study a fast, simple, sensitive/precise and accurate UPLC-MS/MS based method was developed to measure PGE_2 and $PGF_{2\alpha}$ in the cell culture medium, with a limit of quantification 100-times lower for PGE_2 (1 nM vs 100 nM) than the method previously described by Cao *et al* (26). A more sensitive protocol for the quantification of $PGF_{2\alpha}$, which is lower than those described previously (27), was also developed. Nevertheless, in either ST-T1b and HdF cells we could not detect PGF2α. We believe that the method developed and used in this study to investigate the effect of AEA on PG levels in differentiating stromal cells, might be useful also for future works towards the identification of PGs as biomarkers for endometrial receptivity and early pregnancy failure.

As expected, PGE_2 levels were higher in decidualizing stromal cells compared to non-differentiated cells, and this was accompanied by an increase in COX-2 protein levels, in both cell models. Interestingly, AEA and PGs levels are inversely related during differentiation. In order to understand the intricate crosstalk between eCB and PG underlying human differentiation, the effect of AEA on PGE_2 levels was also investigated by the UPLC-MS/MS method developed. The most striking of the present study was the dual effect of AEA in the control of PGE_2 release. In undifferentiated stromal cells, AEA-treatment led to a slight increase of PGE_2 levels, whereas, in cells undergoing decidualization, AEA-inhibited PGE_2 production through a CB1-dependent mechanism. Interestingly, these dual effects of AEA on PGE_2 levels further corroborates the importance of the down-regulated AEA levels in the onset of stromal cell differentiation and/or negative control of decidualization. A previous study reported that eCBs modulate PG production in the fetal membrane in late pregnancy, rather than in maternal tissue explants (28). Yet, while this previous study used term decidual explants, herein we describe AEA-induced effects in cells undergoing decidualization. In support of our findings, we observed that AEA induced a down-regulation in COX-2 protein levels. This effect was also attenuated by CB1 receptor antagonists in HdF cells and totally reversed in St-T1b cells, and directly correlated with AEA effect on PGE_2 levels. Therefore, it is tempting to speculate that AEA effect on PG levels in differentiating cells is due to a corresponding inhibitory effect on COX-2 expression.

Overall, we provide strong evidence that deregulated AEA levels, through CB1 activation, interfere with PGE_2 production and signaling, which may interfere with the onset and progression of stromal cell differentiation. To our knowledge, this is the first study to identify and quantify the crosstalk between eCBs and PGs during human endometrial stromal cell differentiation, and its potential implication in human reproduction. Therefore, these findings provide a molecular framework for the delicate dialogue among eCBs, PGs and stromal cell differentiation, which may be crucial for uterine embryo receptivity, implantation and fertility. In the near future, measurement of eCB and PG concentrations may be translated into clinic benefits, not only to monitor the acquisition of receptive endometrium, but also to diagnose potential pregnancy disorders or infertility.

Acknowledgments

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Supplemental Material

Table 1 – Selective ion monitoring (SIM) parameters and retention times of the analytes.

	PGE2	PGF2α	PGE2-d4
Retention time (min)	1.61	1.71	1.32/1.65
Precursor ion (Da)	351	353	355
Product ions (Da)	**271**, 315	**193**, 247	**275**
Cone energy (V)	40	40	30
Collision energy (V)	30	20	26
Dwell time (ms)	0.3	0.05	0.3

Table 2 – Linearity of the UPLC-MS/MS method, limit of detection (LOD) and limit of quantification (LLOQ).

Analyte	r2	LOD (nM)	LLOQ (nM)
PGE2	0,9994	0,05	1
PGF2α	0,9982	0,5	5

Table 3 – Extraction efficiency of the developed method for PGE_2 and $PGF_{2\alpha}$. Inter-assay and intra-assay reproducibility of the UPLC-MS/MS quantitative analysis. RSD (%), relative standard deviation and extraction efficacy (%) for each analyte was obtained at 3 or 2 different concentrations, respectively for PGE_2 and $PGF_{2\alpha}$.

Analyte	1nM				0,2 µM				2 µM			
	Intra-assay		Inter-assay		Intra-assay		Inter-assay		Intra-assay		Inter-assay	
	RSD (%)	Extraction efficiency (%)	RSD (%)	Extraction efficiency (%)	RSD (%)	Extraction efficiency (%)	RSD (%)	Extraction efficiency (%)	RSD (%)	Extraction efficiency (%)	RSD (%)	Extraction efficiency (%)
PGE2	9	89.98	10	85.05	4	101.06	10	96.40	9	96.60	11	94.36
PGF2α	n.d.	n.d	n.d	n.d.	12	96.50	9	86.75	11	101.55	10	96.83

Manuscript V:

Anandamide impairs endometrial stromal cell turnover: antiproliferative effects and cell death

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Anandamide impairs endometrial stromal cell turnover: antiproliferative effects and cell death

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Abstract

The endocannabinoid system (ECS) has been recognized as a crucial player in pregnancy events such as implantation, decidualization and fetoplacental development. It was previously demonstrated that anandamide (AEA), the main endocannabinoid, induces apoptotic cell death in primary rat decidual cell cultures. In addition, alterations of AEA levels have been implicated in several reproductive disorders. Cyclooxygenase-2 (COX-2) is a major enzyme expressed in endometrium, and its involvement on female reproductive system has evolved along the last years. Recently, COX-2 oxidative metabolism is emerging as a key mediator of AEA-induced effects. In this study we aimed to disclose the role of anandamide in human endometrial stromal cell fate. We found that AEA has an antiproliferative activity through a direct effect on cell cycle progression by inducing G2/M retention and accumulation of binucleated cells. Moreover, AEA triggers mitochondrial apoptotic pathway by causing loss of the mitochondria transmembrane potential, activation of endoplasmic reticulum stress and oxidative stress in a COX-2-dependent manner. Human endometrium is a remarkable dynamic tissue. During the reproductive life, the endometrium undergoes a balanced cellular turnover, between proliferation, differentiation and shedding by apoptosis. Imbalance of endometrial homeostasis is likely associated with pregnancy disorders, infertility and endometrial pathologies. Therefore, we hypothesize that altered AEA levels may deregulate endometrial cell turnover and consequently interfere with cellular events crucial for implantation and decidualization. In conclusion, our findings bring insights on the impact of altered eCBs levels upon human endometrium.

Introduction

The human endometrium is a highly dynamic tissue that undergoes continuous cycles of remodeling. The endometrial stromal cells proliferate and then differentiate, at the secretory phase of the menstrual cycle, into specialized decidual cells in a process named decidualization. Decidualization prepares the uterus for the “implantation window”, the period in which a successful pregnancy can be established through a suitable dialog between the activated blastocyst and the receptive endometrium [1]. In the absence of pregnancy and upon hormonal withdrawal, high cellular turnover in the stromal compartment culminates in menstruation and enables the subsequent tissue regeneration [2]. Apoptosis plays a key role in tissue remodeling and maintenance of cellular homeostasis throughout the menstrual cycle [3]. Cell death is essentially detected at the late secretory and menstruating endometrium, while very little apoptosis was observed during the proliferative phase or at the beginning of the secretory phase [4].

The Endocannabinoid System (ECS) has emerged as a key modulator of multiple physiological and pathophysiological processes, including in a myriad of reproductive events like implantation, decidualization and placental development [5, 6]. Anandamide (AEA) is currently the best studied endocannabinoid (eCB). It is synthesized from membrane phospholipids by the enzyme *N*-acylphosphatidylethanolamine-phospholipase D (NAPE-PLD). After exerting its effects, AEA is hydrolysed inside the cell mainly by Fatty Acid Amide Hydrolase (FAAH), an inner cellular membrane enzyme, into arachidonic acid and ethanolamine. The former is the precursor for prostaglandins production by cyclooxygenase enzyme (COX-2) [7]. Nevertheless, AEA may also be subjected to COX-2 oxidative metabolism, forming prostaglandin-ethanolamides or prostamides (PMEs) [8]. The complexity of the ECS is further supported by the wide range of signaling pathways underlying eCBs-effects. Besides the typical cannabinoid receptors (CB), which are G protein-coupled receptors (GPCRs) [7], AEA may intracellularly activate other targets, like the transient receptor potential vanilloid 1 (TRPV1) ion channel [9], or the peroxisome proliferator-activated receptor (PPAR) [10].

Moreover, lipid-rafts appear to play a role in an AEA-induced signaling cascade independent of receptor pathways [11]. In that way, AEA modulates cell fate-related processes, like cell death, proliferation and differentiation by triggering different pathways, involving mitogen-activated protein kinase (MAPK) kinase, production of ceramide, enzyme expression regulation, induced oxidative stress, and others [7]. Particularly, in AEA-induced cell death it has been described not only the involvement of CB receptors and/or TRPV1 [12-14], but also other receptor independent mechanisms. For instance, in non-tumor Chang liver cells AEA-induced cell death is mediated by ceramide and p38/JNK pathway [15], while in cholangiocarcinoma cells, is dependent upon ceramide synthesis and Fas/FasL localization in lipid rafts [16]. In cytotrophoblast cells, AEA effects involve a crosstalk between intrinsic and extrinsic apoptotic pathways [17]. In primary rat decidual cell cultures, AEA induces cell death through a mechanism partially dependent on CB1 and through COX-2 oxidative metabolism [12, 13, 18]. In addition, AEA-apoptotic cell death in colon and skin cancer cell lines is also mediated by COX-2 metabolism [19, 20].

In humans, AEA levels fluctuate throughout the menstrual cycle through an interplay regulated by FAAH activity, sex steroids and gonadotrophins [21, 22]. In fact, altered AEA levels have been associated with implantation failure, pregnancy disorders and infertility and also with impaired responsiveness of human endometrium to deciduogenic cues. A balanced cellular turnover in the endometrium during menstrual cycle is of key significance, and it is widely accepted that eCBs modulate the choice between cell proliferation, cell death and differentiation. Also, non-differentiated endometrial stromal cells are present in both the endometrium and decidua [23, 24]. Nevertheless, up to date, the role of AEA in human endometrial stromal cells has never been addressed. Thus in this study we studied the role of AEA role on uterine endometrium stromal cells' fate.

Material and methods

Cell culture

The human endometrial stromal cell line St-T1b, obtained from uterine biopsy samples at the time of hysterectomy for benign gynaecological disorders, and immortalized with human telomerase reverse transcriptase (hTERT) [25] was kindly supplied by Dr. Birgit Gellersen (Endokrinologikum Hamburg, Hamburg, Germany). Cells were maintained at 37 °C with 5% CO_2 in DMEM/F-12 medium (Gibco Invitrogen Corporation, Carlsbad, CA, USA) supplemented with 10% charcoal-stripped fetal bovine serum (CT-FBS) (Gibco Invitrogen Corporation, Carlsbad, CA, USA), 1 nM 17-β- estradiol (E_2) (Sigma-Aldrich Corporation, Saint Louis, MO, USA), 1 µg/ml Insulin (Sigma-Aldrich Corporation, Saint Louis, MO, USA) and 2% penicillin-streptomycin (Gibco Invitrogen Corporation, Carlsbad, CA, USA). Initially, cells were incubated for 24 h in completed medium and then experiments were performed in DMEM/F-12 with 1 % CT-FBS.

Cell viability and cell proliferation

Cells were cultured in 96-well plates at a density of 0.5 x 10^4 cells/ well for 24, 48 and 72 h in the presence or absence of AEA (0.1 µM – 50 µM) (Tocris Bioscience, MN, USA). After incubation, MTT (0.5 mg/ml) (Sigma-Aldrich Corporation, Saint Louis, MO, USA) was added to each well and the plate was incubated for 2 h 30 min at 37 °C. The formazan was dissolved by the addition of DMSO: isopropanol mixture (3:1) and quantified spectrophotometrically (540 nm). LDH release in the culture medium was measured by using CytoTox 96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to the manufacturer's protocol. To verify the role of cannabinoid and vanniloid receptors, the antagonists of CB1 (AM251 and AM281; 0.1 µM, Tocris Bioscience, MN, USA) and of TPVR1 (capsazepine; 1 µM) (Tocris Bioscience, MN, USA) were added 30 min prior to AEA. As previously reported, St-T1b cell line does not express CB2 receptor, therefore it was not object of study. Cells were also pre-incubated with two inhibitors of ceramide biosynthesis, L-cycloserine and fumonisin B1 (1 µM, St Cruz Biotechnology, CA, USA) for 30 min and a membrane cholesterol

depletor, methyl-β-cyclodextrin (MCD; 0.5 mM) (St Cruz Biotechnology, CA, USA) for 1 h 30 min. In order to study the involvement of COX-2 and oxidative stress, cells were pre-incubated with selective COX-2 inhibitor, Celecoxib (0.1 μM) (St Cruz Biotechnology, CA, USA), and N-acetylcysteine (NAC 1mM) (St Cruz Biotechnology, CA, USA) 30 min before AEA treatment. At the concentrations used in the study none of the compounds *per se* affected cell viability.

To evaluate the effect of AEA on St-T1b cell proliferation after 48 h of treatment, thymidine incorporation assay was performed. Thymidine incorporation was assessed by the addition of 3H-thymidine (0.5 μCi) (Amersham Biosciences Corporation, NJ, USA) in the last 36 h of the 48 hof AEA- treatment. After a cycle of freezing/defrosting, cellular content was harvested and after addition of a scintillation cocktail, ^{3}H-thymidine incorporation was determined in a scintillation counter (LS 6500, Beckman Instruments, CA, USA). All results are expressed as relative percentage of the untreated control cells.

Morphological studies

Morphological alterations induced by AEA (10 μM) treatment for 48 h were evaluated by Giemsa and Hoechst staining. After treatment, cells plated in 24-well culture plates (1.2 x 10^4 cells/well) were fixed with methanol and stained with Giemsa. For Hoechst staining, cells were exposed to 0.5 μg/ml Hoechst 33342 (Sigma-Aldrich Corporation, Saint Louis, MO, USA) for 20 min and examined under a fluorescence microscope (Eclipse Ci, Nikon, Tokyo, Japan) equipped with an excitation filter with maximum transmission at 360/400 nm and processed by Nikon NIS Elements v 4.0 software.

Cell cycle analysis

After treatment, cells were fixed in 70% cold ethanol and resuspended in 0.5 ml of DNA staining solution (5 μg/ml Propidium Iodide (PI), 0.1% Triton X-100 and 200 μg/ml Dnase-free Rnase A in PBS) (Sigma-Aldrich Corporation, Saint Louis, MO, USA) overnight at 4 °C. DNA content was analysed by flow cytometry based on the acquisition of 40 000 events (with a threshold of 100 000) in a BD Accuri™ C6 (Becton–Dickinson, San Jose, CA, USA) equipped with BD Accuri C6 software. The three fluorescence channels (FL-1, FL-2 and FL-3) and detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale. Debris and aggregates were gated out and cell singlets and doublets were analysed using two-parameter plot FL-2-Area to FL-2-Width of PI fluorescence. The antiproliferative effect was indicated by the percentage of cells in G0/G1, S and G2/M phases of cell cycle.

Determination of caspase -3/-7 activities

The Caspase-Glo® 3/7 (Promega Corporation, Madison, WI, USA) was used to evaluate caspase 3/7 activities according to the manufacturer's protocol. Cells were seeded in 96-well white plates and treated with AEA (10 μM), in the presence or absence of Celecoxib and NAC. Staurosporine (STS, 100 nM) (Sigma-Aldrich Corporation, Saint Louis, MO, USA) was used as a positive control. The resultant luminescence was measured in relative light units (RLU) using a 96-well Microplate Luminometer (BioTek Instruments, Vermont, USA).

Evaluation of mitochondrial transmembrane potential (Δψm)

Mitochondrial transmembrane potential (Δψm) was studied by flow cytometry, using 3,3-dihexyloxacarbocyanine iodide DiOC6(3) at 30 nM for 30 min at 37 °C (Gibco Invitrogen Corporation, Carlsbad, CA, USA) and. After treatment, PI (5 μg/ml) was added prior to flow cytometry analysis to discriminate among

live cells that stain only with $DiOC6(3)^{+}/PI^{-}$, early apoptotic cells that lost the ability to accumulate $DiOC6(3)^{-}/PI^{-}$, and late apoptotic/necrotic cells that stain only with $DiOC6(3)^{-}/PI^{+}$. As positive control, cells were incubated with 15 μM of the mitochondrial depolarizing agent carbonyl cyanide m-chlorophenylhydrazone (CCCP) (Sigma-Aldrich Corporation, Saint Louis, MO, USA). Detectors for forward (FSC) and side (SSC) light scatter were set on a linear scale. Detectors were set on logarithmic scale, the FL-1 was used to measure

DiOC6(3) at green fluorescence and FL-2 and FL-3 to measure PI red fluorescence. Data was analysed using a BD Accuri C6 software based on the acquisition of 20 000 events (with a threshold of 100 000).

Intracellular reactive oxygen species (ROS) measurement

For assessment of ROS production it was used 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA) probe (Sigma-Aldrich Corporation, Saint Louis, MO, USA) at 15 µM. After treatment, cells were washed with PBS and incubated with DCDHFDA for 1 h, at room temperature. As positive control, cells were incubated with phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich Corporation, Saint Louis, MO, USA) at 25 ng/ml. The fluorescence intensity was detected using a Microplate Fluorimeter (BioTek Instruments, Vermont, USA) with excitation and emission wavelengths of 480 nm and 530 nm, respectively. The results were expressed in relative fluorescence units (RFU). For all measurements, basal fluorescence was subtracted.

Western blot

Cells were cultured with AEA in the presence or absence of Celecoxib for 48 h. Cell extracts were prepared in potassium phosphate buffer containing a cocktail of protease inhibitors. Protein concentrations were measured by Bradford assay. Samples (20 µg) were run on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Membranes were then incubated with antibodies against goat-COX-2 (1:100) (sc-1745, St Cruz Biotechnology, CA, USA), rabbit-CHOP-10 (1:100) (sc-575, St Cruz Biotechnology, CA, USA), rabbit-pAkt-123 (1:100) (sc-7985-R, St Cruz Biotechnology, CA, USA) and rabbit-Akt (1:200) (sc-8312, St Cruz Biotechnology, CA, USA) at 4 °C overnight. Membranes were then washed and incubated with goat or rabbit IgG horseradish peroxidase-conjugated antibody (St Cruz Biotechnology, CA, USA) and detected by enhanced chemiluminescence, Western Bright™ ECL and exposed to x-ray film. β-Tubulin (1:500) (H-235, Santa Cruz Biotechnology, CA, USA) was used as a loading control.

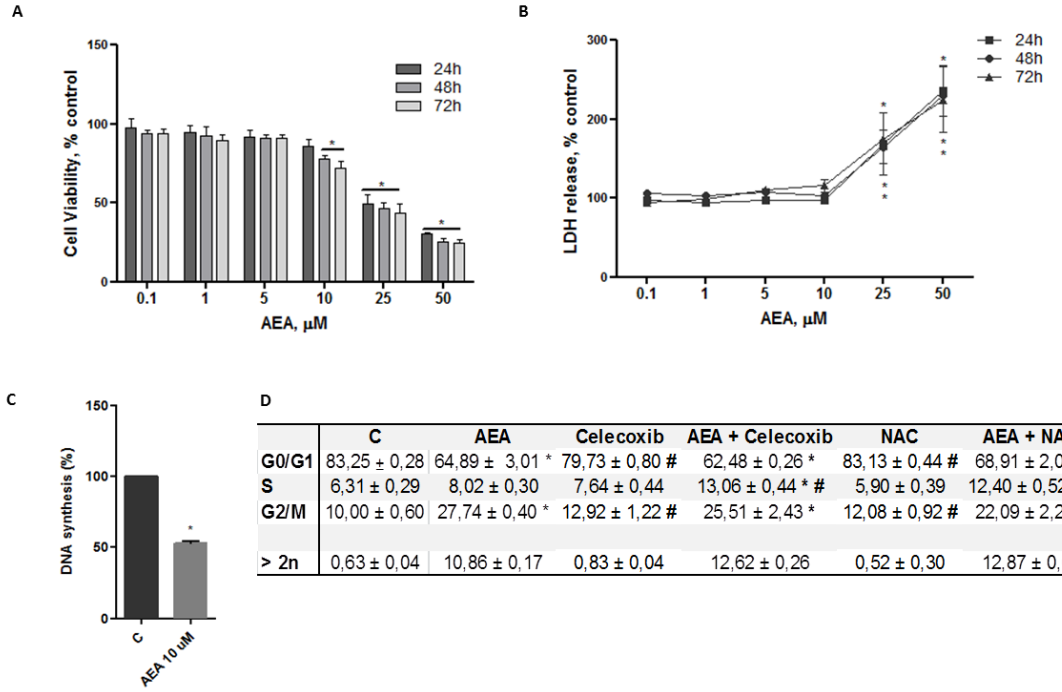
Statistical analysis

Statistical analysis was performed using analysis of variance (ANOVA) followed by Tukey test for multiple comparisons (GraphPad PRISM version 4.0, GraphPad Software, Inc., San Diego, CA, USA). The results are the mean of at least three independent experiments carried out in triplicate. All numerical data are expressed as mean ± SEM and differences were considered to be statistically significant when $p < 0.05$.

Results

AEA-effects in St-T1b cells viability and cell proliferation

To evaluate the effects of AEA in St-T1b cells viability and cytotoxicity, MTT and LDH assays were performed after 24, 48 and 72 h of treatment. As shown in Fig. 1A, it was found a decrease in cell viability for concentrations higher than 5 µM. At 10 µM AEA-induced a significant reduction of viable cells (20%), after 48 and 72 h of treatment. However, after 24 h of treatment, higher concentrations of AEA led to a marked decrease in cell viability, which was accompanied by the release of LDH, indicative of membrane integrity loss (Fig. 1A, B). Since, AEA at 10 µM after 48 h caused a reduction in cell viability without inducing LDH enzyme release, this concentration was selected for the subsequent assays. To analyse whether the reduction in cell viability was due to inhibition of cell proliferation, thymidine incorporation assay was carried out. As shown in Fig. 1C, AEA-induced a drastic decrease in the rate of DNA synthesis (50%). To further explore the underlying mechanism associated with the antiproliferative activity of AEA, it was evaluated its effect on cell cycle progression by measuring DNA content by flow cytometry. In AEA-treated cells, a decrease in the percentage of cells at G0/G1 phase was observed compared to control (64,89 % vs 83,25 %, respectively). It was also found a significant cell cycle arrest of 18 % at G2/M phase compared to control (Fig. 1D).



	C	AEA	Celecoxib	AEA + Celecoxib	NAC	AEA + NAC
G0/G1	83,25 ± 0,28	64,89 ± 3,01 *	79,73 ± 0,80 #	62,48 ± 0,26 *	83,13 ± 0,44 #	68,91 ± 2,07 *
S	6,31 ± 0,29	8,02 ± 0,30	7,64 ± 0,44	13,06 ± 0,44 * #	5,90 ± 0,39	12,40 ± 0,52 * #
G2/M	10,00 ± 0,60	27,74 ± 0,40 *	12,92 ± 1,22 #	25,51 ± 2,43 *	12,08 ± 0,92 #	22,09 ± 2,28 *
> 2n	0,63 ± 0,04	10,86 ± 0,17	0,83 ± 0,04	12,62 ± 0,26	0,52 ± 0,30	12,87 ± 0,03

Fig. 1- Effects of Anandamide in St-T1b cell viability and cell proliferation. (A) AEA at 10 µM induced a decreased of 20 % in cell viability after 48 h. Only concentrations higher than 10 µM caused the release of LDH enzyme (B). (C) Cell proliferation was evaluated by measuring the rate of DNA synthesis. 10 µM AEA-induced a drastic decrease on the rate of DNA synthesis. (D) The study of AEA effects on cell cycle distribution showed a G2/M retention and appearance of binucleated cells. (*p < 0.05 vs. Control; #p < 0.05 vs. AEA).

In addition, it was also observed a drastic increase in the number of bi- or multinucleated cells (11%) upon AEA-treatment. Giemsa and Hoeschst staining further confirmed the presence of binucleated cells (Fig. 2B, D). Through cell counting it was shown a significant increase of 10% in binucleate cells when compared to control (Fig. 2F). Additionally, it was observed that AEA-treated cells exhibited a decrease in cell density and some typical features of apoptotic cell death, such as chromatin condensation (Fig. 2B, D).

AEA-induced cell viability loss in a cannabinoid and vanniloid receptor-independent manner

To further understand the possible involvement of cannabinoid and vanniloid receptors on AEA-induced effects in St-T1b cell viability, cells were pre-incubated with two selective CB1 receptor antagonists (AM251 and AM281 at 0.1 µM) and Capsazepine, a selective antagonist of TPVR1 (1 µM). At these concentrations the compounds *per se* did not affect cell viability.

Moreover, it was found that neither CB1 nor TPVR1 receptor antagonists counteracted the AEA-induced decrease in cell viability (Fig. 3A). In addition, it has been proposed that ceramide production may also be involved in eCB-induced apoptosis. Therefore, St-T1b cells were pre-incubated with two inhibitors of ceramide biosynthesis, L-cycloserine (1 µM), an inhibitor of serine palmitoyltransferase, and fumonisin B1 (1 µM), an inhibitor of ceramide synthase. As shown in Fig. 3A, neither L-cycloserine nor fumonisin B1 were able to revert the effect in cell viability. Also, the inhibitors *per se* did not affect cell viability.

On the other hand, as eCBs may also interact with cell membrane regions enriched with cholesterol, it was shown that the membrane cholesterol depletor, methyl-β-cyclodextrin (MCD at 0.5 mM) reverted the AEA-induced decrease in cell viability (Fig. 3A). As oxidative stress is also associated with eCB induced cell death, cells were pre-incubated with an antioxidant, N-acetylcysteine (NAC 1 mM). In the presence of NAC, the reduction of cell viability was prevented.

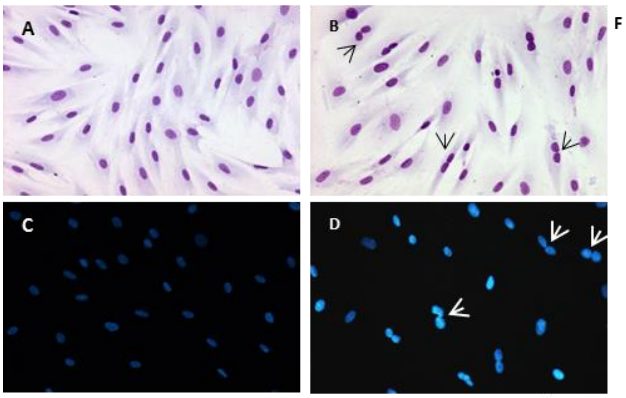


Fig. 2 – Effects of AEA in St-T1b cells morphology. In AEA-treated cells it was found a significant accumulation of binucleated cells by Giemsa staining (arrows) (B) in comparison to untreated cells (A). Höechst staining also supported the presence chromatin condensation and binucleated cells (arrows) in AEA-treated cells (D) when compared to the control (C). (E) Cell counting of binucleated cells (10%) after treatment. (*p < 0.05 vs. Control).

Furthermore, as recent studies evidenced the role of COX-2 oxidativemetabolism mediating AEA activity, the effect of a selective COX-2 inhibitor (Celecoxib) was analysed. Celecoxib (0.1 µM) significantly reverted the loss of St-T1b cells viability (Fig. 3A). As the involvement of COX-2 became apparent, the effect of AEA on COX-2 protein levels was investigated by Western blot. However, upon 48 h of treatment, COX-2 protein levels were not significantly altered in AEA-treated cells (Fig. 3B). To get further insight on the role of Celecoxib and NAC in the AEA-induced antiproliferative effect, it was also investigated their effects in cell cycle progression. Neither Celecoxib nor NAC were able to prevent the cell cycle arrest at G2/M phase, nor the percentage of binucleated cells observed with AEA treatment. Nevertheless, as can be seen in Fig. 1D, it was found a significant S phase accumulation in cells treated with AEA plus Celecoxib (13%) or AEA plus NAC (12%) vs the respective control (7,64% and 5,90%, respectively) or AEA-treated cells (8.02 %).

Oxidative stress, endoplasmic reticulum stress and Δψm loss underlying AEA-induced apoptosis: a role for COX-2 oxidative metabolism To explore the ability of AEA to induce cell death in St-t1b cells, the effects on mitochondrial transmembrane potential (Δψm) loss and the activity of caspase 3/7 were evaluated by flow cytometry and luminescence, respectively. Once the generation of intracellular reactive oxygen species (ROS) may also be implicated with apoptosis, ROS production was assessed by fluorometry. As shown in Fig. 4A, after AEA treatment, it was observed a significant increase in the percentage of depolarized cells (44,68 %) comparing to control (5%). Comparing to the ratio treatment/control, it was also observed that Celecoxib partially reverted the AEA-induced Δψm loss, whereas the antioxidant NAC was not able to counteract this effect (Fig. 4A). In addition, it was detected a significant increase of 28% in the generation of ROS in AEA-treated cells compared to control, an effect counteracted by pre-incubation with Celecoxib and NAC (Fig. 4B).

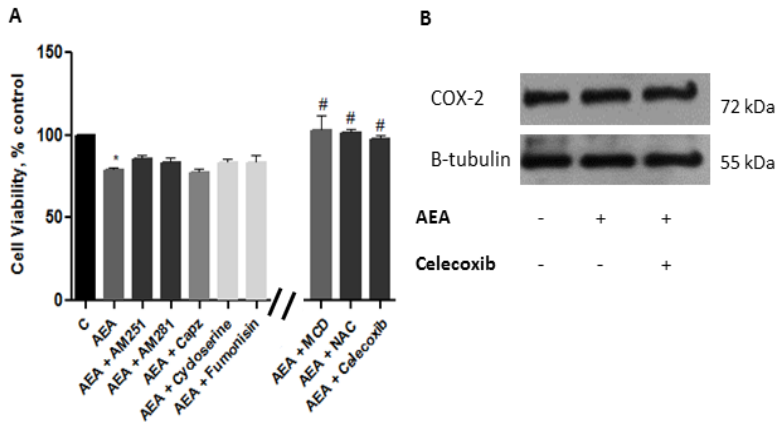


Fig. 3 – The involvement of AEA binding receptors and oxidative metabolism in cell viability. (A) Effect of selective CB and TPVR1 antagonists plus AEA on cell viability. AEA-effects are not mediated through receptor activation. Methyl-β-cyclodextrin (MCD), a membrane cholesterol depletor completely reversed the AEA-induced effect on St-T1b cell viability. Celecoxib, a selective COX-2 inhibitor, and NAC a potent antioxidant were also able to revert the AEA-induced decrease on cell viability. (B) Representative western blot showing that AEA did not significantly affect COX-2 protein expression. (*p < 0.05 vs. Control; #p < 0.05 vs. AEA).

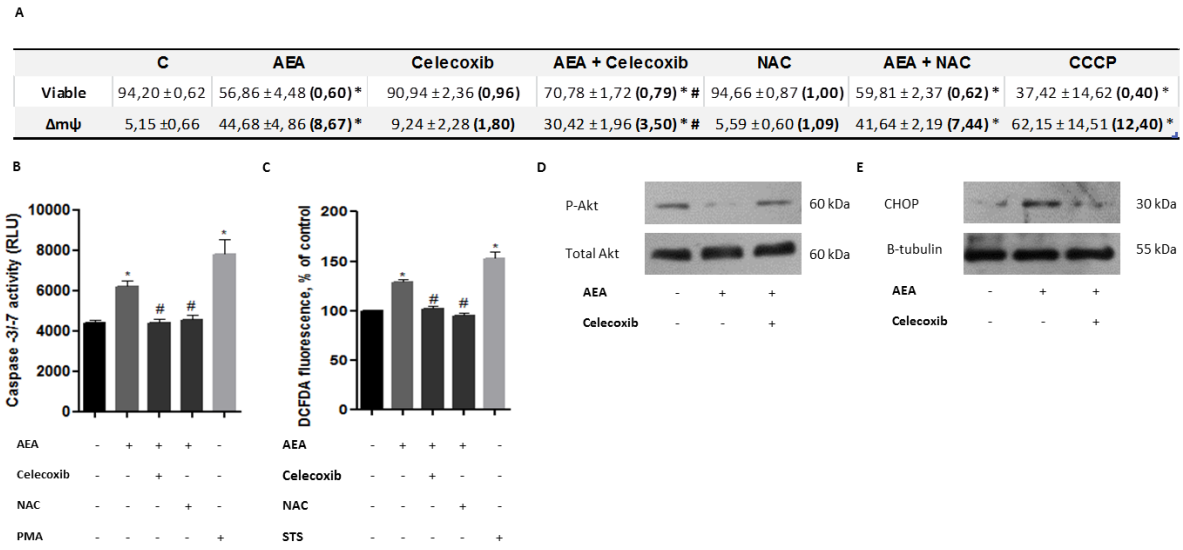


Fig. 4 – Effect of AEA on apoptosis: ΔΨm, ER stress and oxidative stress. (A) Mitochondrial transmembrane potential (ΔΨm) of St-T1b cells treated with AEA (10 μM) with or without Celecoxib and NAC. Only Celecoxib partially reverted the loss of ΔΨm induced by AEA. Data are presented as viable cells and cells with ΔΨm loss. Cells not exposed to AEA were considered control cells. Cells treated with Celecoxib alone were considered control cells of AEA plus Celecoxib. While cells treated with NAC were considered control cells of AEA plus NAC. The ratio treatment/control is presented in bold within brackets. (B) AEA caused a 20% increase of ROS, an effect prevented by pre-incubation with both Celecoxib and NAC. (C) AEA caused a significant increase on the activity of caspase -3/-7, an effect reverted in the presence of Celecoxib and NAC. (D) AEA inhibits phosphorylation of Akt as showed by analysis of protein levels by Western blot. (E) AEA-induced apoptosis involves ER stress, as demonstrated by the analysis of CHOP protein levels which were up-regulated in AEA treated cells, an effect reverted by Celecoxib. (*p < 0.05 vs. Control; #p < 0.05 vs. AEA).

To confirm the presence of apoptosis, the activity of caspase -3/7 was evaluated. AEA caused a significant increase on caspase -3/-7 activity of approximately 20% in comparison to control. Pre-incubation with Celecoxib and NAC showed a significant decrease in activation of caspase activity (Fig. 4C). To further clarify the apoptotic pathway underpinning AEA-effect, Akt phosphorylation was analysed by Western Blot. AEA inhibited the phosphorylation of Akt, an effect that was counteracted in the presence of Celecoxib (Fig. 4D).

To investigate whether endoplasmic reticulum (ER) stress was underlying AEA-induced effect, the protein levels of the cytotoxic ER stress marker, the transcription factor C/EBP (CCAAT/ enhancer-binding protein) homologous protein, known as CHOP, were evaluated by Western blot. Upon 48 h of treatment, in AEA-treated cells it was observed an increase of CHOP protein levels, whereas in the presence of Celecoxib, CHOP levels were similar to control (Fig. 4E).

Discussion

The role of the endogenous cannabinoids, in particular AEA, upon several reproductive events, such as embryo implantation, decidualization, placental development and labour, has been extensively reviewed over the last years [6]. Nevertheless, still little is known about the impact of altered AEA levels on the human endometrial stromal cells. Therefore, in this study a human endometrium immortalized cell line (St-t1b) was employed to unveil the role of AEA upon cell proliferation, cell cycle progression and induction of cell death.

Our findings revealed that AEA-induced a reduction in St-T1b cells viability along with a significant reduction in cell proliferation. The effect in cell viability was independent of CB and TPVR1 receptors, but prevented by MCD, a membrane cholesterol disruptor, thus suggesting that AEA acts intracellularly. Our results also showed that celecoxib, a selective COX-2 inhibitor, and NAC, a potent antioxidant, were able to revert the AEA-induced loss of St-T1b cells viability. In this study, we have further demonstrated that AEA-antiproliferative activity was associated with a cell cycle arrest at G2/M phase and the appearance of bi- and multi-nucleated cells. Since neither Celecoxib nor NAC, were able to prevent these features, it may be suggested that AEA inhibits cell proliferation through a direct effect on cell cycle progression.

Contrary to our results, the antiproliferative effect of AEA in human hepatocellular carcinoma cells [26], human breast and prostate cancer cell lines appears to be dependent on CB receptors [27-29]. Furthermore, in neuroblastoma cells this effect was achieved through a lipid raft-dependent mechanism [30]. However, in St-T1b cells, the AEA-induced G2/M retention and subsequent accumulation of binucleated cells may result from aberrant mitosis and failed cytokinesis, as it has been previosuly suggested for other studies [31, 32]. Moreover, it has also ben reported that defects in mitotic spindle checkpoint, like impaired microtubule depolymerisation, can result in G2/M arrest [33, 34]. Nevertheless, the physiological and molecular significance of this finding has not yet been disclosed and is being object of study. Reinforcing the antiproliferative effect, it was also observed that AEA inhibits phosphorylation of Akt, a kinase of PI3k/Akt pathway, which mediates cell survival and proliferation [35]. Besides associated with an antiproliferative effect, G2/M cell cycle arrest may also be related with enhanced apoptosis [36].

Herein, we further disclosed that AEA triggered the intrinsic apoptotic pathway, supported by the loss of ΔΨm and caspase -3/7 activity, through COX-2 oxidative metabolism. Moreover, the involvement of ROS generation and increased CHOP expression was also shown. Strikingly, it was shown that AEA-apoptotic events were independent of receptor activation but rather occur through a mechanism dependent on COX-2 oxidative metabolism. In fact, the oxidative metabolism of AEA by COX-2 is a source of bioactive lipid messengers, named prostamides. The latter are emerging as mediators of AEA-induced apoptosis in tumorigenic keratinocytes [20], and in colorectal carcinoma cell lines [37]. Moreover, in primary cultures of rat decidual cells, AEA-induced cell death through the main AEA-oxidative derivative, prostamide E2 [18]. Also, in non-melanome skin cancer, AEA-induced ER stress and apoptosis are mediated by oxidative stress and COX-2 oxidative metabolism [20, 38]. As expected, Celecoxib and NAC were able to inhibit AEA-induced ROS production and caspase 3/7 activity.

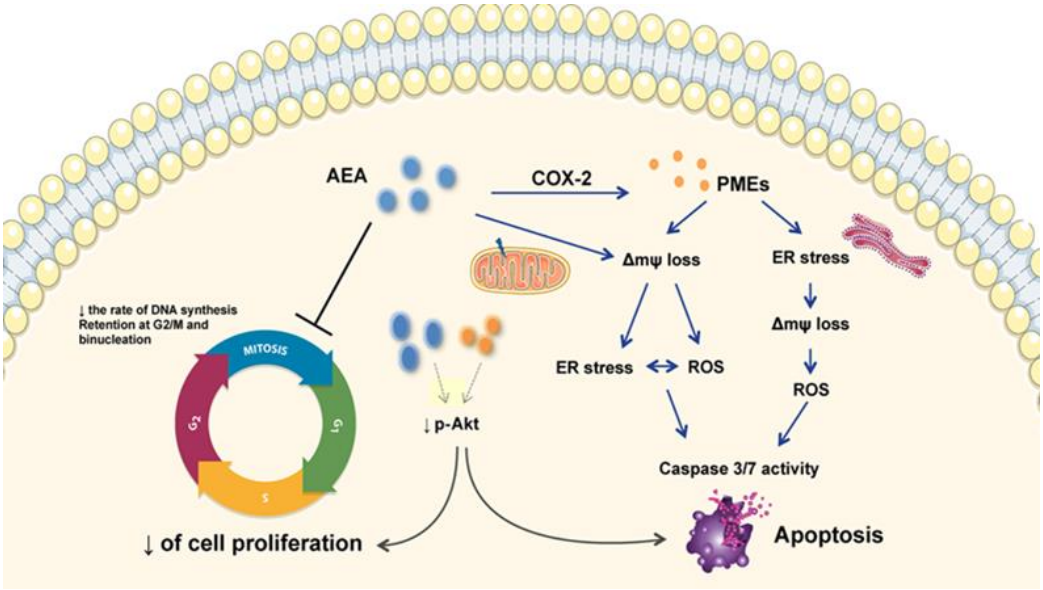


Fig. 5 – Proposed mechanism of AEA action on human endometrial stromal cells. AEA inhibits endometrial stromal cell proliferation, as shown by the reduction on the rate of DNA synthesis, probably associated with Akt inhibition. This antiproliferative effect is mediated through cell cycle arrest at G2/M phase and accumulation of binucleated cells. COX-2 is a major enzyme expressed in the human endometrium. Hence, under the studied conditions, it is suggested that AEA may be subjected to COX-2 oxidative metabolism giving rise to Prostamides (PMEs). In the one hand, PMEs may lead to ΔΨm loss which may induce ER stress and ROS production. On the other hand, it may also be that PMEs induce ER stress with consequent loss of ΔΨm and ROS generation. These signaling pathways will ultimately lead to caspase -3/7 activation and cell death. PI3k/Akt pathway may also be involved in apoptosis. Hence besides AEA, PMEs may also inhibit Akt phosphorylation. Moreover it appears that AEA may also cause ΔΨm loss directly. AEA – blue circles, PMEs – orange circle.

Concerning the decline of ΔΨm, NAC was not able to revert this effect, suggesting that changes in mitochondrial dysfunction may lead to oxidative damage and ROS production. On the other hand, Celecoxib only partially prevented the loss of ΔΨm, indicating that AEA may, at least in part, directly modulate mitochondrial depolarization. Accordingly, it has been recently reported the ability of (endo)cannabinoids, but also their derivatives, to control mitochondrial function directly [39]. Although the molecular events linking ΔΨm, ROS and the ER stress pathway are still elusive, evidence connecting these distinct processes is increasing. Recently, the mitochondrial production of ROS, as a result of ER stress–induced Ca^{2+} release or impairment of endogenous antioxidant defence mechanism, has gained considerable importance in cell death [40-42]. In addition, CHOP may also down-regulate Bcl-2 and induce the translocation of Bax to mitochondria leading to apoptosis [43]. On the other hand, mitochondrial dysfunction may also contribute to ER stress [44].

Although ER stress can lead to ROS production, redox status or generation of ROS may also directly or indirectly (or both) affect ER homeostasis [40]. In response to ER stress, the unfolded protein response (UPR), an intracellular signal transduction pathway may be activated to promote cell survival or induce cell death through CHOP up-regulation [45]. Consistent with our results, other groups have demonstrated that ER stress may regulate AEA-induced apoptosis [46, 47]. On the one hand, CHOP-induced apoptosis may be mediated through inhibition of the PI3K-Akt signaling pathway [48, 49]. On the other hand, the association with Akt inactivation, Bad dephosphorylation and mitochondrial dysfunction and apoptosis has also been addressed [50]. Hence, we further unveil the mechanisms associated AEA-induced cell death, through an inhibitory effect on Akt activation via COX-2 dependent mechanism. Herein we provide strong evidences on the role of COX-2 oxidative metabolism in AEA-induced cell death in endometrial stromal cells.

In this regard, it has been recently reported that COX-2 mRNA expression is highest during the proliferative phase. Therefore, it is tempting to speculate that in an endometrial environment with high COX-2 expression, elevated AEA levels may represent a niche for prostamide production which may mediate AEA-apoptotic cell death. In conclusion, we describe the impact of AEA in human endometrial stromal cell turnover and propose the cellular pathways that may be involved (Fig. 5). Two major breakthroughs are highlighted: the AEA-COX-2 oxidative metabolism underlying-ER stress and oxidative stress, mitochondrial dysfunction and apoptosis and it is also disclosed, for the first time, an antiproliferative effect of AEA mediated through a direct effect on cell cycle progression.

In this way, we also reinforce the relevance of balanced eCBs signaling upon uterine environment, since exacerbated AEA levels may interfere with endometrial cell turnover leading to implantation failure, inappropriate decidual response and/or miscarriage.

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CHAPTER III

General Discussion and Final Remarks

Discussion and general conclusions

The highly dynamic nature of endometrium is a remarkable feature of reproductive biology and a major challenge of human infertility. Endometrial stromal cells undergo cyclical waves of proliferation and differentiation, which are followed by programmed cell death and endometrial repair. These events are imposed after a miscarriage, parturition and menstrual shedding. A network of cytokines, hormones, growth-factors and other molecules contributes to endometrial homeostasis and remodelling. In the past few years, endocannabinoids (eCBs) have been recognized as important mediators of several aspects of human physiology. Anandamide (AEA), the main eCB, is a key player in reproduction involved in embryo development and transport, implantation, uterine receptivity, pregnancy maintenance and labor. Hence, a deregulation of AEA signalling has been associated with poor implantation, recurrent miscarriage, pregnancy disorders, and infertility (333). In this way, fatty acid amide hydrolase (FAAH) and cyclooxygenase-2 (COX-2), the degrading enzymes of AEA may be considered important controllers of these reproductive events.

Previous studies have focus on the role of anandamide in rat decidual remodelling. Furthermore, a dual effect of AEA in primary rat decidual cell cultures has been reported. While at lower concentrations AEA induced cell death by a cannabinoid receptor-1 (CB1)-dependent mechanism, higher doses caused dramatic changes in cell viability probably due to a cytotoxic interaction with cellular membranes (65). Endocannabinoids derive from cell membrane phospholipid (PL) precursors. Their levels are maintained by integral membrane enzymes and their biological actions are mediated through cell surface receptors. Therefore, membrane integrity and phospholipid composition are expected to interfere in eCB signalling. In this study, a novel understanding on the mechanisms underlying AEA effects on membrane phospholipid dynamics during rat decidual remodelling process was provided. Herein, the membrane phospholipid profile of rat primary decidual cells was characterized for the first time using a mass spectrometry based lipidomic approach. In addition, it was shown that AEA treatment caused an increment in the total PL content. Interestingly, at the molecular level, the main changes were observed in phosphatidylserine, phosphatidylinositol and cardiolipin relative content, which was correlated with the reported AEA-induced apoptosis in rat decidual cells (65). These changes may result from a cellular adaptation and reorganization of membrane phospholipidome to AEA-induced effect or rather represent a natural consequence of apoptosis. Furthermore, it was reported an increase in the relative content of PL bearing long chain fatty-acids with high degree of insaturation, in almost all molecular PL species. This fact is of particular importance in AEA-induced signalling and metabolism. On one

hand, the high degree of polyunsaturation may influence the function as well as the stability of membrane proteins, such as the CB receptors or the eCBs-metabolic enzymes (382). Besides, membrane fluidity may also be affected and, as so AEA diffusion through plasma membranes. In addition, lipid rafts, which are microdomains enriched in cholesterol, sphingolipids and glycosphingolipids are involved in the modulation of eCBs and CB1-mediated apoptosis (227). Therefore, alterations in membrane composition of lipid rafts are also likely to disrupt AEA transduction pathways. In conclusion, altered lipid composition of plasma membranes may interfere not only with AEA metabolism and uptake, but also with the enzymatic cascade triggered by AEA, directly or indirectly related with CB receptor activation. On the other hand, as previously referred, AEA cytotoxic effects on rat decidual cells may reflect direct interactions with membranes, which may also explain the changes observed in this study (65). Hence, the characterization of the phospholipidome of decidual cells may, in the future, bring insights in the pathophysiology of early pregnancy-related complications associated with disruption of the endocannabinoid system (ECS). In fact, disturbances on lipid metabolism has been associated with some pregnancy disorders, such as pre-eclampsia (383).

Although the precise mechanisms by which eCBs influence reproduction are uncertain, COX-2 is thought to have a major role. COX-2 is increasingly being recognized as a “metabolic keeper” of eCB levels in several biological processes, such as neuroprotection, obesity, inflammation, apoptosis and cell differentiation (384). Therefore, the impact of the oxidative metabolism by COX-2 in AEA-induced decidual cell apoptosis was investigated. Regardless being constitutively expressed in rat decidual tissue, it was found that AEA caused a time-dependent stimulation on COX-2 expression, whereas FAAH expression was not affected. It was further demonstrated that selective inhibitors of COX-2 oxidative metabolism prevented all the previously reported apoptotic features induced by AEA in primary cultures of rat decidual cells, such as chromatin condensation, presence of apoptotic bodies, caspase-3/-7 and -9 activation, loss of mitochondrial transmembrane potential ($\Delta\Psi m$) and increase in reactive species of oxygen (ROS) (65). A remarkable finding of this work was the elucidation of the physiological relevance of the COX-2 oxidative metabolism over AEA during the decidual remodelling process. Although the formation of COX-2-derived metabolites of AEA, named prostamides (PMs), has been known for long, their role on several human pathophysiological events is only recently being explored (384). In this work, PME_2 and $PMF_{2\alpha}$ were identified as endogenous mediators operating in decidual cells. In particular, it was described the molecular pathway involved in the pro-apoptotic effect of PME_2 in rat decidual cells, which was similar to the one described for AEA.

It was previously reported that, in rat decidual cells, AEA induced apoptosis through p38 activation and that CB1 antagonist partially attenuated this effect, suggesting that multiple pathways may modulate this eCB effects (65, 213). In this study, it was further demonstrated that AEA-induced p38 phosphorylation was followed by COX-2 up-regulation, but this effect was independent of CB1 activation. In fact, it has been postulated that eCBs may directly modulate phosphorylation of MAPK kinases under the regulation of cell fate boundaries between proliferation and apoptosis in several biological systems (29). Interestingly, it was also observed that AEA in the presence of a FAAH inhibitor induced phosphorylation of p38 after a short term exposure, while long term treatment reduced p38 activation. Nevertheless, in the presence of the FAAH inhibitor, COX-2 expression was drastically elevated, as well as PME_2 levels. Although the molecular basis of such an observation is still elusive, it may indicate that excess levels of AEA or of its oxidative metabolites, mimicked by FAAH inhibition may trigger a negative feedback loop of p38 phosphorylation, as suggested in other studies (385, 386). In addition, it was also verified that this effect involved NF-kB pathway, which has also been reported by other authors (387, 388). In rats, COX-2 expression is increased upon endometrial stromal cell differentiation, and PGs are crucial for this process (371). In this study, COX-2 metabolism was described as a metabolic controller of AEA decidual signalling and tone. Moreover it was unraveled a novel role for PME_2 as a mediator of cell death during rat decidual remodelling process. Thus, we hypothesize that a disruption of FAAH activity, and/or aberrant eCB levels, may favor COX-2-mediated oxidation of AEA and overwhelm the decidual regression through the formation of pro-apoptotic PMs.

Decidualization occurs during the secretory phase of the menstrual cycle and prepares, each month, a receptive uterus for an eventual pregnancy. In fact, impaired decidualization, as a result of poor hormone responsiveness and/or impaired endometrial stromal cell proliferation and/or differentiation, can be associated with implantation failure, infertility, miscarriages, and pregnancy disorders, such as pre-eclampsia or intrauterine growth retardation and premature birth (389). As it was already referred, it is well established that a complex network of molecules coordinates endometrium differentiation, in which endocannabinoids and prostaglandins (PGs) may be central mediators. Nevertheless, the knowledge regarding the role of eCBs and PGs on human reproduction is scarce and provided mainly from studies in animal models. In rodents, it has been postulated that a tight regulation of *in situ* levels of AEA are indispensable for early pregnancy events.

In this work, for the first time, it was demonstrated that both non-differentiated and differentiated human endometrial stromal cell line (St-T1b) and human decidual fibroblasts (HdF) derived from term placenta, possess the full ECS biochemical machinery. Contrary

to HdF, which expressed both receptors, St-Tb1 cells only expressed CB1 receptor. Interestingly, while NAPE-PLD expression was not affected, the expression of FAAH, at mRNA and protein levels, was up-regulated during the decidualization process. This is consistent with the recognized role of FAAH as a metabolic controller of AEA uterine levels (390). Moreover, it was found that in decidualizing cells, AEA levels dramatically decreased compared to non-differentiated cells, suggesting that this eCB may be important to maintain stromal cells in an undifferentiated state. In fact, it was previously referred that during the menstrual cycle, AEA achieves the lowest levels during the midsecretory/luteal phase which coincides with the occurrence of the decidualization process (360, 364). Based on these observations, it was further explored the role of AEA during human decidualization process *in vitro* in both cell models. In this regard, it was verified that AEA decreased the levels of the genes encoding for IGFBP-1 and PRL, recognized as biochemical markers of decidualization, and these effects were reversed by CB1 blockade. These findings are in agreement with a previous study by Fonseca *et al.* (2015) showing that, in rats, AEA also impairs stromal cell differentiation (239).

Several lines of evidences point that COX-2 derived PGs may also be important players in this process (366). Since an intimate dialogue between eCBs and PGs appears to be involved in major reproductive events, the influence of AEA on COX-2 expression and PGE_2 release was investigated. In fact, the potential use of PGs as biomarkers for endometrial receptivity and early pregnancy failure has increasingly been explored (375), though limitations in PG measurement greatly hamper the profiling of these molecules in human samples. To overcome this limitation, a fast, simple, with a good linearity, sensitive/precise and accurate UPLC-MS/MS based method was developed and validated to measure PGE_2 and $PGF_{2\alpha}$ in cell culture medium. This new method allowed the identification of both PGs with lower quantification and detection limits compared to others described in the literature (391, 392). As expected, upon endometrial stromal-derived cell differentiation, COX-2 protein expression and PGE_2 levels were increased. The inverse correlation pattern between AEA and PGE_2 levels observed during decidualization indicated that low levels of AEA and increased PGE_2 levels may be essential to the onset of the decidual program. In addition, another major finding of this study was the dual effect of AEA in the regulation of PGE_2 production. While in non-differentiated cells, AEA-induced a slight increase of PGE_2 levels, in cells undergoing decidualization COX-2 expression and PGE_2 release were diminished. Collectively, these findings highlight AEA as a novel mediator of human decidualization process in a mechanism dependent on CB1 receptor and through deregulation of COX-2 expression and PGE_2 production. Moreover, this work greatly contributes to the knowledge that PGE_2 and AEA tone have the potential to be exploited as novel diagnostic biomarkers of infertility.

Parallel to these observations, it was found that AEA inhibited endometrial cell proliferation in both non-differentiated and in cells undergoing decidualization, which was not accompanied by programmed cell death. The ability of AEA to suppress cell proliferation was previously described in various tumor cell types. For instance, in human breast cancer and prostate cancer cell lines it appears to be dependent of CB receptors (203, 204, 206), while in neuroblastoma cells this effect was achieved through a lipid raft-dependent mechanism (210). A remarkable finding of this work was the appearance of polyploidy upon AEA-treatment in both models and cell types, non-differentiated and differentiated stromal-derived cells which was independent of CB1 activation. Although the biological significance of this fact is not yet disclosed, evidences support that this effect may derive from failed cytokinesis and/or impaired microtubule depolymerisation, causing a G_2/M blockade, as it has been suggested in other studies (393-397). In fact, in rodents, endoreduplication (polyploidy) of endometrial stromal cells is well established (288, 291) and appears to result from a mitotic cycle without cytokinesis. This fact was associated with the limited life-span of decidual cells and may ensure the increased protein synthesis required during feto-placental development (289). In human decidualization, the presence of bi-nucleated cells has been reported by Tang *et al.* in 1993 (295), and is a topic of increasing recognition in the literature. However, their significance has not been disclosed. Noteworthy, was the ability of AEA to induce bi-nucleation in both non-differentiated and differentiated stromal-derived cells. Curiously, the latter are genetically programmed for the development of polyploidy.

Reinforcing the antiproliferative effect of AEA, it was also observed an inhibition of Akt phosphorylation, a kinase involved in cell survival and proliferation (398). Interestingly, diminished Akt phosphorylation has been associated with inhibition of actin polymerization and consequently cytokinesis failure, appearance of multi-nucleated cells and cell growth inhibition (399). Hence, this further supports the premise that AEA may directly impair cytokinesis. Taking into account our results showing that AEA induced changes on the lipid composition of rat decidual cell membranes, recent studies reported that cells actively regulate and modulate their lipid composition and localization during cytoplasmic division. In fact, it has been suggested that the metabolism of specific phospholipids, such as phosphatidylinositol and phosphatidylserine may be involved in the stepwise assembly and dynamics of regulatory complexes and cytoskeletal structures during cytokinesis (400, 401). In this way, deregulation of membrane phospholipid synthesis and integrity maintenance can have a dramatic impact on the process of mitosis and cytokinesis (402).

Under physiological conditions, high AEA plasma levels are found in the follicular/proliferative phase. During this phase, a balanced cellular turnover, involving cell proliferation and apoptosis, mainly regulated by estrogen is responsible for the

endometrial regeneration, and consequently provides a privilege matrix for stromal cell differentiation. The impact of AEA on human endometrial stromal cells has never been addressed, yet we hypothesized that eCBs may also be important in coordination with hormones and cytokines, in the regulation of the proliferative phase of menstrual cycle. As referred, it is widely accepted that AEA mediates several cellular events ranging from cell proliferation, differentiation and cell death, dependent on the biological context and cell type. Moreover, the discrepancies of AEA induced effects depending on its concentration have been described. For instance, while low doses of AEA induced cell death in rat decidual cells, high doses caused a dramatic decrease of cell viability associated with the involvement of necrosis (65). On the other hand, eCBs were also reported to impair human cytotrophoblast cells syncytialization without affecting cell viability, while also being modulators of apoptosis (91, 240). In addition, in rat, it was postulated that while at low levels AEA accelerated trophoblast differentiation and outgrowth, at high levels induced opposite effects (57).

In the experimental conditions used to study the role of AEA on non-differentiated St-T1b cells, contrary to the above mentioned, AEA caused a decrease in cell viability which was associated with a significant reduction in cell proliferation and apoptotic cell death. In addition, these events were independent of CB1 activation. Nevertheless, it was observed that a membrane cholesterol disruptor counteracted the reduction on St-T1b cell viability caused by AEA. This finding, besides indicating an intracellular induced-effect, also underlies the importance of the phospholipid membrane integrity in regulating AEA biological roles. In addition, Celecoxib, a selective COX-2 inhibitor, was also able to prevent the loss of St-T1b cell viability, evidencing the involvement of oxidative metabolism on AEA-induced cell death. In this way, it was also verified that AEA triggered the mitochondrial apoptotic pathway, causing loss of $\Delta\Psi m$, inducing endoplasmic reticulum (ER)-stress, ROS generation and caspase activation in a COX-2-dependent manner. However, Celecoxib only partially prevented the loss of $\Delta\Psi m$, suggesting that besides the oxidative metabolites of AEA, the latter may also directly affect mitochondrial function. In fact, this capacity has been recently reported not only for (endo)cannabinoids but also for their derivatives (403). In addition, it was demonstrated that alterations in mitochondrial transmembrane potential preceded ER-stress and ROS production, since *N*-acetylcysteine, (NAC), an antioxidant, was not able to prevent loss of $\Delta\Psi m$. In fact, in decidual cells, ER-stress has been associated with oxidative stress as a potential mechanism of early pregnancy loss (404). Thus the ability of AEA to increase ROS production and induce ER stress must be further investigated, though this has already been referred in rat decidual cells and trophoblast cells (56, 213). In addition, altered redox status on fetal-maternal interface has been associated with some pregnancy

disorders, such as pre-eclampsia (404-406). Moreover, endometrial shedding by apoptosis and menstruation has been associated with stimulation of COX-2 expression and $PGF_{2\alpha}$ production through ROS-induced NF-kB activation (407). Thus, this study provides strong evidences on the role of anandamide and its COX-2-derivatives in human endometrial stromal cells. Since non-differentiated endometrial stromal cells are present in both the endometrium and decidua (408, 409), it may be suggested that abnormal AEA signalling, may interfere with important biological processes, and therefore impair the differentiation capacity of endometrium as well as the progression of the decidualization process. Moreover, in an environment privileged by a heightened COX-2 expression, endometrium may represent a niche for potential production of PMs that, similar to the referred above in rat model, may mediate AEA-apoptotic cell death and interfere with endometrial remodelling.

Final Remarks

In conclusion, it was demonstrated that, depending on the environment conditions, AEA controls endometrial cell proliferation, differentiation and apoptotic cell death in a specific manner, mainly involving a particular pattern of COX-2 regulation. In this sense, this work provides further insights on the promiscuity of AEA in controlling endometrial stromal cells' fate. It is suggested that AEA may activate several signalling pathways, in which individual effects might be overloaded, and the final outcome depend on the development stage of the endometrium, the environmental context and the molecular targets.

In rat decidual cells, anandamide increased COX-2 expression, via p38 activation in cells undergoing apoptosis. Furthermore, PME_2 oxidative metabolite was proposed as a novel mediator in endometrial remodelling.

In human endometrial stromal-derived cells, besides characterization of the ECS, anandamide, the main eCB, was proposed as a negative modulator of cell differentiation, associated to a decrease in COX-2 protein levels and PGE_2 release, through a CB1-dependent mechanism. Another major breakthrough of this work is the important role of COX-2-mediated pathways of the AEA metabolism in stromal endometrial cells. Hence, along with the conventional FAAH enzyme, they may be considered as metabolic gatekeepers of the AEA tone on human endometrium. In this regard, our findings further supported that eCBs and PGs may be potential biomarkers of human endometrium receptivity and infertility. In addition, it was reported for the first time, the anti-proliferative effect of AEA in endometrial stromal cells associated with the development of polyploidy, probably by a direct interference with cytokinesis.

Understanding the molecular signature that underlies the role of AEA on endometrial stromal cells further elucidates the cellular and molecular basis that governs decidualization. Therefore we suggest that AEA may underpin the complex molecular network composed by hormones, cytokines and growth factors that orchestrates human decidualization. Nevertheless, these observations also point out that exacerbated AEA levels, whether resultant from deregulation of metabolic enzymes or *Cannabis sativa* consumption may impair basic cellular process involved in endometrial remodelling which may ultimately account for aberrant decidual response, pregnancy loss, miscarriage and infertility.

CHAPTER IV

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